2014 | Faculty of Medicine and Life Sciences

DOCTORAL DISSERTATION

Exploiting epigenetic mechanisms and dendritic cells to treat multiple sclerosis

Doctoral dissertation submitted to obtain the degree of doctor of Biomedical Science, to be defended by

Kristof Thewissen

Promoter: Prof. Dr Niels Hellings | UHasselt / tUL
Co-promoter: Prof. Dr Piet Stinissen | UHasselt / tUL

D/2014/2451/62

“The man who follows the crowd will usually get no further than the crowd. The man who walks alone is likely to find himself in places no one has ever been.” (Alan Ashley-Pitt)

Members of the jury

Prof. dr. I. Lambrichts, Hasselt University, Diepenbeek, Belgium, chair

Prof. dr. N. Hellings, Hasselt University, Diepenbeek, Belgium, promotor

Prof. dr. P. Stinissen, Hasselt University, Diepenbeek, Belgium, copromotor

Prof. dr. V. Somers, Hasselt University, Diepenbeek, Belgium

Prof. dr. J. Hendriks, Hasselt University, Diepenbeek, Belgium

Dr. H. Slaets, Hasselt University, Diepenbeek, Belgium

Prof. dr. Z. Berneman, UZA, Antwerp, Belgium

Prof. dr. G. van Loo, VIB inflammatie-researchcentrum, Ghent, Belgium

Prof. dr. J.W. Voncken, Maastricht UMC+, Maastricht, The Netherlands

Table of contents

Table of contents I
List of figures V
List of Tables VII
List of Abbreviations VIII

Chapter 1. Introduction and aims 1

1.1 Basic concepts of MS 2
1.1.1 Diagnosis and clinical course 2
1.1.2 Genetic and environmental factors 3
1.1.3 Immunopathogenesis of MS 5
1.1.4 Animal models 9
1.1.5 Therapies 9
1.2 Dendritic cells in MS 12
1.2.1 DC origin and subsets 12
1.2.2 Functional role of DC in keeping the immune balance 15
1.2.3 Evidence for the involvement of DC in MS pathogenesis 16
1.2.4 DC-based therapy 17
1.3 Epigenetics and MS 20
1.3.1 Epigenetic mechanisms 20
1.3.2 Evidence for the role of epigenetic mechanisms in MS 21
1.3.3 Exploiting epigenetic mechanisms to treat MS 25
1.4 Aims of the study 26

Chapter 2. Circulating dendritic cells of multiple sclerosis patients are proinflammatory and their frequency is correlated with MS-associated genetic risk factors 29

ABSTRACT 30
2.1 Introduction 31
2.2 Materials and methods 33
2.2.1 Study population 33
2.2.2 Isolation and stimulation of leukocytes 35
2.2.3 Flow cytometry 35

2.2.4 In vitro migration assay 35
2.2.5 Cytokine ELISA 36
2.2.6 HLA-DRB1*1501 and IL-7Rα genotyping 36
2.2.7 Statistics 36
2.3 Results 38
2.3.1 pDC, but not cDC frequency is decreased in the peripheral blood of CPMS patients as compared to RRMS patients and healthy controls 38
2.3.2 HLA-DRB1*1501 and polymorphisms in the promoter region of IL-7Rα affect the frequency of circulating DC 38
2.3.3 Circulating DC of MS patients have a migratory and costimulatory phenotype indicative of an inflammatory disease state 41
2.3.4 DC of MS patients behave differently after TLR stimulation 44
2.4 Discussion 47

Chapter 3. Tolerogenic dendritic cells generated by in vitro treatment with SAHA are not stable in vivo 51

ABSTRACT 52
3.1 Introduction 53
3.2 Materials and methods 55
3.2.1 Mice 55
3.2.2 DC generation, culture and treatment 55
3.2.3 Cell surface phenotype analysis 56
3.2.4 Myelin phagocytosis 56
3.2.5 Cytokine ELISA 57
3.2.6 STAT signaling 57
3.2.7 EAE induction and treatment with DC 57
3.2.8 MOG specific immune cell proliferation assay 58
3.2.9 Statistics 58
3.3 Results 59
3.3.1 SAHA treatment delays the generation of bone marrow derived $CD11c^+$ DC 59
3.3.2 SAHA-treated $CD11c^+$ DC fail to upregulate costimulatory molecules after LPS stimulation 60

3.3.3 SAHA-generated CD11c+ DC downregulate the expression of molecules involved in apoptosis-induction, migration and TLR signaling 61
3.3.4 SAHA-treated CD11c+ DC produce less pro-inflammatory cytokines after LPS stimulation 62
3.3.5 Endocytosis of myelin and MHC-II expression is diminished after 10d SAHA 63
3.3.6 SAHA effects coincides with reduced STAT1 signaling and are independent of STAT6 signaling 65
3.3.7 SAHA treatment did not enhance clinical potency of MOG-pulsed CD11c+ DC in EAE 66
3.3.8 Ex vivo SAHA-generated tolerogenic DC phenotype is not stable 67
3.4 Discussion 69

Chapter 4. The DNA methylation inhibitor Decitabine completely blocks autoimmune mediated CNS demyelination 73

ABSTRACT 74
4.1 Introduction 75
4.2 Materials and methods 77
4.2.1 Mice 77
4.2.2 EAE induction and treatment with DAC 77
4.2.3 Determine frequency of immune cells 77
4.2.4 MOG specific immune cell proliferation assay 78
4.2.5 Flow cytometry 78
4.2.6 Immunohistochemistry 78
4.2.7 Real-time PCR 79
4.2.8 Statistics 82
4.3 Results 83
4.3.1 DAC inhibits the development of EAE 83
4.3.2 DAC treatment reduces the number of immune cells present in primary and secondary lymphoid organs and inhibits priming of MOG reactive T cells 84
4.3.3 DAC treatment reduces the number of Th17 cells in the spleen of EAE mice 86

4.3.4 Foxp3 expression is increased by DAC in $CD4^+$ T cells 87
4.3.5 DAC reduces the upregulation of genes important in driving inflammation 88
4.4 Discussion 91

Chapter 5 Summary and general discussion 95

Chapter 6 Nederlandse samenvatting 113

Reference list 119
Curriculum vitae 143
Bibliography 144
Dankwoord 147

List of Figures

Figure 1.1	Differentiation of the various DC subsets from bone marrow	14
Figure 1.2	Possible mechanism to target dendritic cells and shift DC towards tolerogenic properties	18
Figure 2.1	Circulating pDC are decreased in CPMS patients	38
Figure 2.2	Blood circulating cDC are decreased in HLA-DRB1*1501^{+} MS patients	40
Figure 2.3	Circulating pDC in the blood are decreased in MS patients not carrying haplotype 2	40
Figure 2.4	Expression levels of CD86 are elevated in cDC of MS patients	41
Figure 2.5	Expression levels of migratory markers are increased during MS	43
Figure 2.6	cDC and pDC of MS patients show an increased migratory potential towards chemokines specific for CCR5 en CCR7	44
Figure 2.7	Representative example of intracellular cytokine staining of TLR-stimulated cells	46
Figure 3.1	Morphology and purity of cell cultures	56
Figure 3.2	10 days SAHA treatment limits the differentiation of CD11c^{+} DC	60
Figure 3.3	SAHA treatment of 10 days blocks the costimulatory upregulation of CD80 and CD86 after LPS stimulation	61
Figure 3.4	SAHA treatment of 10 days reduces the expression of FasL, CCR5, TLR4 and TLR9 on CD11c^{+} DC	62
Figure 3.5	DC cell cultures treated with SAHA produce less pro-inflammatory cytokines after LPS stimulation	63
Figure 3.6	CD11c^{+} DC-generated for 10d with SAHA take up less myelin and show reduced MHC-II levels	65
Figure 3.7	SAHA blocks maturation of CD11c^{+} DC through diminished phosphorylation of STAT1 (pY701) and independent of STAT6 phosphorylation (pY641)	66
Figure 3.8	MOG-pulsed DC reduced disease symptoms independent of SAHA treatment during 3 consecutive days	67

Figure 3.9	SAHA treatment of 10 days does not induce a permanent inhibition of CD80 or CD86 expression after SAHA removal	68
Figure 4.1	DAC treatment inhibits the development of EAE	84
Figure 4.2	No increases in immune cell numbers are detected in lymph nodes or spleens of DAC treated mice, whereas the cell frequency in the thymus is reduced compared to DMSO controls	86
Figure 4.3	DAC treatment reduces the presence of Th17 cells in the spleen	87
Figure 4.4	Foxp3 expression is increased in $CD4^+$ T cells during DAC treatment	88
Figure 4.5	DAC reduces the upregulation of genes involved in driving inflammation and migration	89
Figure 5.1	Summary of the most important alterations in the DC compartment of MS patients as reported in this thesis	102

List of Tables

Table 1.1 Overview of the effects of tolerogenic DC therapies in EAE, the animal model of MS 19

Table 1.2 Possible epigenetic biomarkers in MS 23

Table 2.1 Clinical details of the patients recruited into the study 34

Table 2.2 Clinical and genetic background of the patients recruited into the study 37

Table 2.3 IL-12p70 and IFN-α secretion after TLR ligation 45

Table 4.1 Primer sequences for RT-PCR 80

List of Abbreviations

AML	Acute myeloid leukemia
ANOVA	Analysis of variance
BBB	Blood brain barrier
BDCA	Blood DC antigen
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDC	Conventional or myeloid DC
CDP	Common DC precursor
CFA	Complete Freund's adjuvant
CIS	Clinically isolated syndrome
CLEC	C-type lectin-domain
CNS	Central nervous system
Cpm	Counts per minute
CPMS	Chronic progressive MS
CSF	Cerebrospinal fluid
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CX3CR	CX3C chemokine receptor
CXCL	Chemokine (C-X-C motif) ligand
CXCR	C-X-C chemokine receptor
DAC	5-aza-2'-deoxycytidine or Decitabine
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dpi	Days post immunization
DTR	Diphteria toxin receptor
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-barr virus
EDSS	Expanded disability status scale
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

FBS	Fetal bovine serum
FCS	Fetal calf serum
Foxp3	Forkhead box P3
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Granulocyte-macrophage progenitor
hap	Haplotype
HAT	Histone acetyltransferase
HC	Healthy control
HDAC	Histone deacetylase
HLA	Human leukocyte antigen
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HSC	Hematopoietic stem cell
iDC	Immature DC
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's modified dulbecco's media
iNOS	Inducible nitric oxide synthase
IQ	Imiquimod
IRF8	Interferon regulatory factor 8
LP	Lymphoid committed precursor
LPS	Lipopolysaccharide
Ly6C	Lymphocyte antigen 6C
MBP	Myelin basic protein
MDP	Monocyte, macrophage and dendritic cell precursor
MDS	Myelodysplasic syndrome
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
miRNA	microRNA
MIP	Macrophage inflammatory protein
MLP	Multi-lymphoid progenitor
moDC	Monocyte-derived DC
MOG	Myelin oligodendrocyte glycoprotein
MP	Myeloid committed precursor
MRI	Magnetic resonance imaging

mRNA	Messenger RNA
MS	Multiple sclerosis
N.D.	Not determined
NF-κB	Nuclear factor-kappaB
NO	Nitric oxide
PAD2	Peptide arginine deiminase 2
PBS	Phosphate buffered saline
PD-L1	Programmed death-ligand 1
PMBC	Peripheral blood mononuclear cell
pDC	Plasmacytoid DC
PML	Progressive multifocal leukoencephalopathy
PPMS	Primary progressive MS
Pre-cDC	preclassical dendritic cell
RANTES	Regulated on activation, normal T cell expressed and secreted
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPL13a	60S ribosomal protein L13a
RPMI	Roswell park memorial institute medium
RRMS	Relapsing remitting MS
S1P	Sphingosine-1-phosphate
SAHA	suberoylanilide hydroxamic acid
SEM	Standard error of the mean
SI	Stimulation index
SNP	Single-nucleotide polymorphism
SPMS	Secondary progressive MS
STAT	Signal transducer and activator of transcription
tDC	Tolerogenic DC
TGFβ	Transforming growth factor β
Th	T helper cell
TipDC	TNF-iNOS producing DC
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TNFRSF1A	Tumor necrosis factor receptor superfamily member 1A
Treg	Regulatory T cell

TSDR	Treg-specific demethylated region
TSLPR	Thymic stromal lymphoprotein receptor
UPN	Unique patient number
UV	Ultaviolet
VLA-4	Very Late Antigen-4
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation

1

Introduction and aims

1.1 Basic concepts of MS

1.1.1 Diagnosis and clinical course

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS). It is characterized by inflammation, axonal degeneration and gliosis (1, 2). MS has a high prevalence rate with more than 100-200 MS cases per 100 000 individuals in the Western world. This disease affects more women than men (2:1 ratio) and becomes clinically apparent between the ages of 20 and 40, making MS the most common neurological disease in young adults (3, 4).

1.1.1.1 Diagnosis

Jean Martin Charcot was the first to describe '*la sclérose en plaque*' in 1868 (5). Since then the field has seen many adaptations of proposed diagnostic criteria for MS (6-8). Until now, there is no single laboratory test for the diagnosis of MS (8). Initially the diagnostic criteria for MS were based on clinical features. The Schumacher criteria stated that 2 clinical relapses separated in time and space are needed in patients between 10-50 years old and no other explanations for associated symptoms must be present (9). Later, the presence of oligoclonal bands in the cerebrospinal fluid (CSF) and delayed responses of the visual and auditory evoked potentials were added by Poser to the criteria (10). The current criteria, the McDonald criteria, further incorporate magnetic resonance imaging (MRI) with clinical and laboratory assessment (11). These diagnostic criteria include an initial clinical presentation with symptoms typical for an MS attack followed by carrying out several tests, such as CSF analysis to measure immunoglobulin concentration, which are increased in more than 90% of MS patients, electrophysiological studies which show delayed latencies of visual, somatosensory and auditory evoked potentials and blood tests to exclude other conditions that mimic MS (6-8, 12).

1.1.1.2 Clinical course

MS can follow different clinical courses and usually starts with an initial clinically isolated syndrome (CIS) in 85% of MS patients (13). This is followed by recurrent episodes of neurological impairment due to various periods of inflammation and demyelination interchanged with episodes of recovery defined as relapsing remitting MS (RRMS). In this early stage, perivascular inflammatory lesions are

found which results in the hallmark of MS namely demyelinating plaques. This inflammation leads to damage of oligodendrocytes and myelin, which surrounds and insulates axons. In this way, the relay of neuronal signals is disrupted in the affected regions (1, 2, 14). Overtime, in 25-40% of MS patients, RRMS proceeds into a chronic progressive form where the emphasis lies more on neurodegeneration and less on inflammation. In this SPMS phase, there is a further accumulation of neuronal damage and associated disability becomes permanent and irreversible (6, 15). While the majority of patients follow an RR course of the disease, 10-15% of MS patients develop a progressive course, termed primary progressive MS (PPMS), from the onset although relapses and remissions can be superimposed in some chronic MS patients (7). The heterogeneity of the MS course and affected regions lead to a variety of symptoms including impairment of bladder and bowel function, muscle weakness, sensory disturbances, visual loss, cognitive impairment, fatigue and paralysis of extremities (3, 16, 17).

1.1.2 Genetic and environmental factors

MS is a complex and heterogeneous disease where the exact etiology remains unknown. However it is generally assumed that disease develops as a result of an interplay between genetic, environmental factors and immune disbalances.

1.1.2.1 Genetic predisposition

Studies have shown that first-, second- and third-degree relatives of MS patients are at higher risk (3-5%) to develop MS in comparison with the general population (0.1%) (18). Moreover higher concordance rates were shown in monozygotic twins (30-50%) as compared to 5% in dizygotic twins. These studies indicate that genetic components play a role in MS susceptibility (19-21). Genome-wide association studies have identified several MS-associated loci, with the human leukocyte antigen (HLA) class II genes accounting for 14%–50% of the genetic burden. More specifically HLA-DRB1*1501 carriers have a three to four times higher relative risk of developing MS (20, 22-24). Also other immune-related genes were found to be associated with MS including interleukin 2 receptor alpha (IL2RA), IL7RA, major histocompatibility complex class I (MHCI), CD25, CD127, CD58, C-type lectin-domain family 16 member A (CLEC16A), tumor necrosis factor receptor superfamily member 1A (TNFRSF1A), CD6 and interferon

regulatory factor 8 (IRF8) (25-33). Although these associations are weaker than the HLA-II alleles, multiple genes contribute to the increased risk in this complex disease.

1.1.2.2 Environmental factors

Although genetic susceptibility can partly explain the increased risk in developing MS, the discordance in monozygotic twin studies provides evidence that other interacting factors contribute to the disease. Data from immigration studies reveals that migrants who moved from a high incidence MS region to a lower incidence show a decreased risk (34). Genetic susceptibility cannot explain such changes in a short time frame. This strongly highlights the influence of environmental factors. It is generally established that MS prevalence increases with geographic latitude in an incomplete distribution model. For example MS frequency is lower in Canada than expected based on ambient temperature and latitude (35, 36). Consequently epidemiological studies have identified several environmental risk factors including infections, smoking, sunlight exposure, vitamin D and nutrition (4, 37). The solar exposure hypothesis provides one of the strongest correlations of latitude with MS. Sunlight intensity and ultraviolet (UV) radiation can influence the MS incidence. It is shown that increased exposure is protective against MS, probably via attenuating T helper cell 1 (Th1) mediated responses (4, 38, 39). Moreover UV radiation converts cutaneous 7-dehydrocholesterol to pre-vitamin D_3. This pre-vitamin converts subsequently towards the active form calcitriol, which has immune modulating effects, by inducing regulatory T cells (40-43).

Several studies have suggested an association between viruses and MS, for example chlamydia pneumoniae, varicella-zoster virus, human herpes virus-6 and cytomegalovirus (4, 44-46). The strongest association with MS can be found with the Epstein-barr virus (EBV) (47, 48). These viruses can play their role in the pathogenesis of MS by two means; bystander activation or molecular mimicry. In the first, autoreactive T cells are activated by inflammatory molecules generated against the infection, whereas in mimicry the pathogen resembles an endogenous peptide. In this way, an immune response is started against the self-peptide (37). As an example, it is shown that a peptide sequence of EBV is homologous to the myelin basic protein (MBP) peptide, claiming evidence for the involvement of viruses in the pathogenesis of MS (4). Other environmental influences, such as

smoking and nutrition, can evoke pathology in genetically susceptible individuals and are associated with MS disability and relapses (49-51).
To summarize, it is clear that environmental as well as hereditary factors are involved in determining the risk of developing MS. Recent evidence suggests that epigenetic changes can provide the missing link between external exposures and inherited genetic systems. In paragraph 1.3 a detailed description of epigenetic mechanisms and their involvement in MS is given.

1.1.3 Immunopathogenesis of MS

The CNS was long considered to be immune privileged owing to the blood brain barrier (BBB). However, studies have established that immune cells can migrate to the CNS through multiple routes (52-54). Leukocytes are able to enter the brain via; the blood-to-subarachnoid space through leptomingeal vessels, blood-to parenchymal perivascular space through the BBB, blood-to-CSF through the choroid plexus, meningeal spaces and the ependymal lining ventricles (53, 54). Under physiological conditions trafficking of cells is low and they rarely invade the parenchyma. They encounter perivascular antigen-presenting cells involved in immune surveillance of the CNS (55, 56). However during disease, leukocyte infiltration is increased and inflammatory reactions are started to clear the insult. When inflammation goes on uncontrolled, like in MS, irreversible damage to resident cells occurs. An overview of the current models and key players in the pathogenesis of MS is given in the following subsections.

1.1.3.1 Classical outside-in model vs alternative inside-out model

In the classic view of MS, immune dysregulation plays an important role in the pathogenesis. In this outside-in model, lymphocytes with encephalitogenic potential escape regulatory mechanisms in the periphery of genetically susceptible individuals and are activated. During activation, encephalitogenic lymphocytes upregulate migration molecules and home to the CNS by crossing the BBB. Several studies describe that immune surveillance takes place in the CNS and that autoreactive cells are present in healthy persons (52-54, 57, 58). The question arises what causes the transition from physiological to pathological autoimmunity in the CNS. Two factors play a role in this transition phase. First, the loss of immune tolerance which normally eliminates autoreactive cells by inducing anergy or apoptosis, receptor downregulation or editing and/or control by regulatory cells.

Second, autoreactive lymphocytes can be activated due to nonspecific activation by bacteria or viruses, as previously described owning to bystander activation or molecular mimicry. Once inside the CNS, pathogenic T cells are reactivated by myelin antigens in a two-step process. Primed lymphocytes encounter CD11c-expressing antigen presenting cells in the perivascular space and become reactivated. This reactivation causes stimulation of CD11b microglia by release of pro-inflammatory cytokines. These cytokines further facilitate the recruitment of other inflammatory cells and leakage of antibodies and other plasma proteins into the CNS. The cascade of events causes damage to the myelin producing cells, specifically oligodendrocytes, and the myelin itself (59, 60). Damage done to the myelin sheats induces axonal loss, eventually leading to a conduction block and neurodegeneration.

In contrast to this model with a primary autoimmune etiology, the inside-out model proposes that the initial trigger of MS starts in the CNS. Oligodendrocytes and myelin are presumed to be targeted by cellular degeneration, leading to the release of debris that is highly autoantigenic, like citrullinated MBP. In a next phase a secondary inflammatory reactions is launched causing additional substantial damage to the CNS (59, 61, 62).

1.1.3.2 Key players in MS pathogenesis

MS is largely considered to be a $CD4^+$ T helper 1 (Th1)-mediated disease (63, 64). Studies have shown that activated myelin-reactive $CD4^+$ T cells are present in the peripheral blood and CSF of MS patients (65, 66). Moreover their presence was demonstrated in MS lesions, predominating in acute lesions. In healthy individuals only nonactivated myelin-specific T cells are identified in the blood. Myelin-specific T cells isolated from the CSF of MS patients have an increased expression of the interleukin (IL) 2 receptor which is consistent with an activated or memory phenotype (63, 67). The typical cytokines of Th1 cells such as IL-2 and TNFα are found within active demyelinating lesions (68). Moreover, the MS-associated genetic risk factor HLA-DRB1*1501 is linked with T cells. This HLA conformation has been found to be more effective in presenting MBP peptide to $CD4^+$ T cells (69-71). Myelin-reactive T cells from MS patients are less susceptible to regulatory mechanisms such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) blockade. Further conclusive results came from observations from the animal model of MS, more specifically experimental autoimmune encephalomyelitis

(EAE). This disease is induced by immunizing animals with myelin components and results in a primarily Th1 mediated disease. Adoptive transfers of isolated myelin-specific T cell clones from EAE animals into naïve recipient animals causes a similar demyelinating disease, leaning evidence to the fact that Th1 cells play a central role in the pathogenesis of MS (64, 72). Recently, studies have changed the conventional view of MS as a Th1 driven autoimmune disease by the discovery of Th17 cells (73-75). IL-23 together with IL-6 and transforming growth factor β (TGFβ) are required for the development of Th17 cells (76, 77). From EAE studies it was revealed that IL-23 is the critical regulator of the disease instead of IL-12 (78). $CD4^+$ Th17 cells are able to cross the BBB in EAE animals and transfer of Th17 cells induces a more severe EAE course compared to adoptive transfer of Th1 cells (74, 79, 80). Although Th17 cells are shown to disturb and cross the BBB in MS patients, the exact role of Th17 cells and IL-17A in MS is unclear (74). However it can be postulated that Th1 and Th17 cells work syngeneic with each other to drive neuroinflammation.

Whereas Th1 and Th17 cells are effector cells, regulatory T cells are needed to maintain self tolerance. Regulatory T cells modulate the immune response by inhibiting proliferation and repressing the cytokine production of various activated immune cells, like $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages and dendritic cells (DC) (81, 82). In MS, more specifically RRMS, regulatory T cells are dysfunctional. The compromised function of regulatory T cells leads to uncontrolled autoreactive T cells activation (83-87).

Besides $CD4^+$ T cells and a disturbed function of regulatory cells, several studies have proven that $CD8^+$ cells are also involved in MS. Post-mortem brain samples and CSF examination of MS patients have revealed that $CD8^+$ T cells are more abundant in lesions and undergo clonal expansion more frequent than $CD4^+$ T cells do (88-90). Moreover, autoreactive $CD8^+$ T cells recognize MBP and can be found at a higher frequency than myelin-specific $CD4^+$ T cells in lesions. Myelin-specific $CD8^+$ cytotoxic T cells produce the proinflammatory chemokines macrophage-inflammatory protein-1α&-1β, IL-6 and CXCL10 and Biddison and colleagues showed that soluble products of this cell type attract myelin-specific T cells (91). Together these studies demonstrate that $CD8^+$ T cells play a role in the recruitment and retention of myelin-specific $CD4^+$ T cells as well as contributing to the damage in the CNS.

Although MS is observed as a T cell-mediated disease, several studies support the fact that B cells are involved in the pathogenesis (92). Firstly, autoantibodies against myelin components such as MBP and myelin oligodendrocyte glycoprotein (MOG) were detected in MS lesions (93-95). Moreover, as already mentioned in the section about diagnosis, oligoclonal bands are found in the CSF of 95% of MS patients (96). In addition, it was demonstrated that 50% of MS patients have deposits of antibodies in active demyelinating lesions (97). Prineas et al. identified receptor-mediated endocytosis of immunoglobulin (Ig) G bound to myelin in MS lesions (98). Besides the presence of antibodies in CSF and lesions, clonally expanded B cells were identified in the CSF and within perivascular lesions (99). Germinal center-like structures can be found within the meninges of some MS patients. These studies provide evidence that B cells are present and chronically active in the CNS (96, 99). Rituximab treatment, a monoclonal antibody against CD20 expressed on B cells, has proven to halt disease progression and improve clinical outcome, confirming the role of the humoral immune system in MS (100, 101).

Myeloid cells such as macrophages, microglia and DC play a role in MS pathogenesis. Dendritic cells are reviewed in depth in section 1.2. The involvement of macrophages in MS was confirmed by Brück and colleagues. They observed the presence of macrophages filled with myelin in MS lesions (102, 103). Macrophages can damage myelin and surrounding cells by production of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), IL-1 and IL-6. For example, TNF-α causes oligodendrocyte apoptosis which leads to an increase in demyelination and a rise in the vulnerability of axons to other factors (104). Another group of mediators produced by macrophages and microglia are reactive oxygen species (ROS) and nitric oxide (NO). Although cells have several antioxidant mechanisms against low concentrations of ROS, macrophages produce these radicals at a high concentration during inflammation. In this manner, antioxidant mechanisms cannot compete with the abundance of radicals and therefore ROS cause oxidative damage to proteins, lipids and nucleic acids (105). Studies have shown that superoxide radicals are increased in serum of MS patients and can induce DNA damage in MS patients leading to neurodegeneration (106-108). Also NO products are present in serum and CSF of MS patients and in active MS and EAE lesions (105, 109). It needs to be mentioned that macrophages have a dual role in MS and also possess anti-inflammatory properties. This was

supported by findings of Boven et al. which demonstrate that myelin-laden (foamy) macrophages display several markers involved in anti-inflammatory responses. Furthermore, alternative activated macrophages are involved in the scavenging of debris, promoting tissue remodelling and repair (110).
Lastly, astrocytes are the most abundant cell type in the CNS and were long considered to provide only a supportive function for neurons. By now it is clear that astrocytes are involved in a variety of functions, such as blood flow regulation, ion and water homeostasis, regulation of BBB function, myelination, neurotransmission and higher cognitive functions (111, 112). In disease, astrocytes can modulate CNS inflammation by contributing to glial scarring (astrogliosis) and promoting inflammation through secretion of detrimental factors like NO and ROS. On the other hand, astrocytes are also important limiting CNS damage by producing neurotrophic factors and are reported to becrucial for potentiating myelin repair (113, 114).

1.1.4 Animal models

Our current understanding of the mechanisms and role of the various immune cells in MS comes from animal models. An animal model of demyelinating diseases that is commonly used is experimental autoimmune encephalomyelitis (EAE) (63, 115).
In EAE, the disease is primarily mediated by peripheral myelin-reactive Th1 and Th17 cells which cause a demyelination in the CNS. EAE is induced in several ways depending on the animal species and involved methods. Between different techniques the pathological outcome and clinical presentation can vary and this makes us able to study different aspects of MS.
Methods of EAE induction range from active immunization of animals with myelin proteins to passive transfer of autoreactive T cells or transgenic animals which spontaneously develop inflammatory disease in the CNS. EAE in these animals is characterized by disturbed motor function and ascending paralysis starting in the tail towards forelimbs (116-120). Because EAE mimics many of the clinical and immunological aspects of MS, it is therefore widely used as a model to study the mechanisms behind MS and to test new therapeutic strategies.
Although animal models are indispensable to study complex immune-mediated demyelination diseases like MS, these models only resemble certain aspects of the disease and cannot always be extrapolated to human disease. Therefore

results of animal studies have to be critically evaluated and only be used to investigate certain features of the disease.

1.1.5 Therapies

No other neurological disease has seen more progression in the development of immunotherapeutic strategies than MS (121-124). It started with the first FDA-approvement of interferon-β1b (Betaseron™/Extavia™) in 1993 and since then 10 more drugs are developed and approved by the FDA.

The first generation of therapeutics consists of type 1 interferon-β (IFN-β) and glatiramer acetate (Copaxone™). The type 1 IFN-β consists of IFN-β1a (Avonex™ and Rebif™) and IFN-β1b (Betaseron™/Extavia™). The beneficial effects come from their anti-inflammatory effects. Moreover IFN-β reduces BBB disruption by decreasing the production of matrix metalloproteinases (125, 126). IFN-β also decreases antigen presentation, has modulatory effects on costimulation, increases expression of IL-10 and suppresses proliferation of Th1 cells shifting the immune response towards anti-inflammatory actions (123, 127-129). Glatiramer acetate is a synthetic polymer consisting of random sequences of four amino acids, namely L-Tyrosine, L-glutamate, L-alanine and L-lysine. Given its resemblance to MBP, it is suggested that glatiramer acetate competes with myelin antigens to be presented by MHC class II molecules. Glatiramer acetate further induces regulatory T cells and sustains the proliferation of Th2 cells and $CD8^+$ suppressor T cells. Other effects include bystander suppression of inflammation and release of neurotrophins (122, 130-134).

Second line therapeutics are mitoxantrone (Novatrone™), natalizumab (Tysabri™) and fingolimod (Gilenya™). Mitoxantrone is a general immunosuppressive drug and the only agent approved to treat SPMS. It intercalates with DNA and also suppresses DNA repair. In this way proliferating immune cells are suppressed and undergo apoptosis. However the use of the drug is limited because of the higher chances of inducing cardiomyopathy and a delayed treatment related acute leukemia (123, 124, 135, 136). Natalizumab inhibits the transendothelial migration of leukocytes to the CNS. The mechanism of action is a humanized monoclonal antibody against the α4 chain of the α4β1 integrin (VLA-4), which is expressed on all leukocytes except neutrophils. An uncommon side effect of this treatment is the occurrence of progressive multifocal leukoencephalopathy (PML) due to the infection of oligodendrocytes by JC virus

(122-124, 137, 138). Fingolimod is a sphingosine-1-phospate (S1P) receptor modulator. It acts as an agonist and donwregulates the receptor S1P expression. Via this manner it induces sequestration of lymphocytes into the lymph nodes which make them unable to invade the CNS and initiate in situ responses (122, 139, 140). Fingolimod also promotes the neuroprotective effects of microglia (141).

Other emerging therapies are still in clinical trials such as for example alemtuzumab (Campath-1H) and dimethyl fumarate (123, 124). Alemtuzumab depletes cells expressing CD52 and induces the production of neurotrophic factors by reconstituted autoreactive T cells. This is suggested to reduce neuroinflammatory damage to the CNS. Dimethyl fumarate reduces the migration of activated leukocytes into the CNS and is presumed to activate antioxidative pathways (123, 124). Phase 3 clinical trials with patients treated with these drugs show lower rates of sustained disability (improved Expanded disability status scale (EDSS) score), reduce annual relapse rates and a lower number of lesions on MRI (142-147).

Although a large number of therapeutics exist, we are still far from developing a cure for MS. These therapeutics show differences ranging from administration routes and frequencies of injections to tolerability, adverse effects and major toxicities. Moreover, the long-term effects of the newest strategies remain uncertain. Additionally, these drugs target predominantly immune dysfunction and are therefore clinically beneficial in RRMS patients. The limited evidence of efficiency in progressive disease is indicative for the involvement of other pathophysiological mechanisms such as mitochondrial dysfunction, exhaustion of remyelination processes and degeneration of axons due to abnormal axonal excitability (121). A better understanding of the progressive phase would lead to development of new therapeutics and intervening early in this phase, as in the case with RRMS, will lead to a significant better clinical outcome.

1.2 Dendritic cells in MS

"Its salient features are its large, contorted, refractile nucleus and its long cytoplasmic processes, which contain many large, spherical mitochondria. Both nucleus and cytoplasm stain weakly with basic dyes, and the cell has none of the morphologic features of active endocytosis, i.e., membrane ruffling, pinocytic vesicles, abundant lysosomes, phagocytosed objects. In the living state in vitro, its cytoplasmic processes are continually elongating, retracting, and reorienting themselves, leading to a wide variety of cell shapes. The term, dendritic cell, would thus seem appropriate for this novel cell in vitro" (148). These are the descriptive words in the paper of Steinman and Cohn in 1973 which first identified DC in the mouse spleen. Since then, an abundance of studies appeared that addressed the role of DC as important regulators of the immune response. In the following sections, important topics with regard to DC are further reviewed.

1.2.1 DC origin and subsets

DC are a highly specialized population of leukocytes of the innate immune system involved in antigen uptake and presentation and are essential to the induction of immunity and tolerance (149, 150). DC are a heterogeneous population of hematopoietic cells that can be found throughout the body. For example spleen, lymph nodes, kidneys, gut and skin are populated by different types of DC (151, 152). Even immune privileged zones, such as the CNS, have DC surveying the environment for pathogens. In this case, DC reside in the CSF, meninges and perivascular zones, while no DC are found in the brain parenchyma (153). Even though no concrete data exist about the exact turnover rates of DC, it is widely accepted that homeostasis of DC relies on the continuous production of DC out of the bone marrow (151). In humans, it is known that DC arise from granulocyte-macrophage progenitors (GMP) and multi-lymphoid progenitors (MLP) from the bone marrow (154, 155). Still, identification of early DC precursors in peripheral blood is difficult since $CD34^+$ hematopoietic stem cells already express MHC-II molecules and further complicates the elucidation of the developmental history of DC in humans. Classification of differentiated DC is complex since several subtypes exist which vary in their origin, lifespan and anatomical location. Differences in expression of Toll-like receptors (TLR), cytokine receptors and cytokines as well as in migratory potential point to different functions in the

induction and regulation of the immune response. For simplicity, four distinct categories have been distinguished (figure 1.1): conventional or myeloid DC (cDC), plasmacytoid DC (pDC), monocyte-derived DC (moDC) and Langerhans cells (156, 157).

Conventional DC are subdivided into $CD1c^+$ ($BDCA\text{-}1^+$) and $CD141^+$ ($BDCA\text{-}3^+$) cDC and can be found in blood, lymph nodes, spleen and non-lymphoid tissue such as skin, liver, lung and gut. cDC are considered to be the conventional inducers of primary T cell responses (149, 158). $CD1c^+$ cDC express both CD11b and high levels of CD11c. In contrast $CD141^+$ ($BDCA\text{-}3^+$) cDC lack CD11b and only express low levels of CD11c but selectively express CLEC9A (156, 159, 160). Based on these phenotypical differences both subtypes are likely able to perform different functions (159).

pDC are $CD11c^-$ and characterized by the expression of CD303 (BDCA-2), CD304 (BDCA-4) and CD123 (156). They are blood and lymph node circulating cells specialized in the production of large amounts of type 1 interferons in response to viral infections. pDC are inefficient in priming T cells and rather induce T cell anergy and the development of regulatory T cells in the steady-state. This function originates from their limited costimulatory and endocytic capacity and low expression of MHC-II molecules. Moreover secretion of IFN-α by pDC shapes the immune response by influencing cDC function (156, 157, 161-163). Furthermore pDC are able to sense skin injuries and promote wound healing (164).

Monocyte-derived inflammatory DC are seen as a reservoir used in case of an emergency. When a danger signal is present, monocytes are rapidly recruited at the site of inflammation and upregulate genes involved in specialized antigen presentation mechanisms. This is needed to quickly counter the attack of the pathogen and in this way these inflammatory moDC support the work of cDC (156, 157). Development of DC out of monocytes was only demonstrated *in vivo* by two mice studies. Serbina et al. showed that inflammatory moDC, also referred to as TNF-iNOS producing "Tip" DC, play an important role in pathogen clearance (165), whereas the second study by Leon et al. recognized moDC as essential to promote early-pathogenic specific T cell responses (166). To date, the differentiation of monocytes to moDC could not be demonstrated in humans.

A last specialized subtype of DC are the **Langerhans cells**. These cells are found within the epidermis of the skin and mucosa where they provide a first barrier against invading pathogens. Langerhans cells follow a distinct development

pathway as they originate from a locally present Ly6C$^+$ myelo-monocytic precursor cell and depend on TGF-β signaling. What makes these cells special is their high expression levels of MHC-II molecules in non-pathological circumstances, making them ideally suited to promote immunity when the skin or mucosae are breached by infections (157, 167, 168). Recently, a role in maintaining immune tolerance was added to the classic function of Langerhans cells (169).

To conclude, several subsets within the DC network orchestrate specific roles in immunosurveillance, migration and antigen presentation. The plasticity between these subtypes provides evidence that DC are excellent in regulating and fine tuning the immune response in both pathologic and physiologic circumstances.

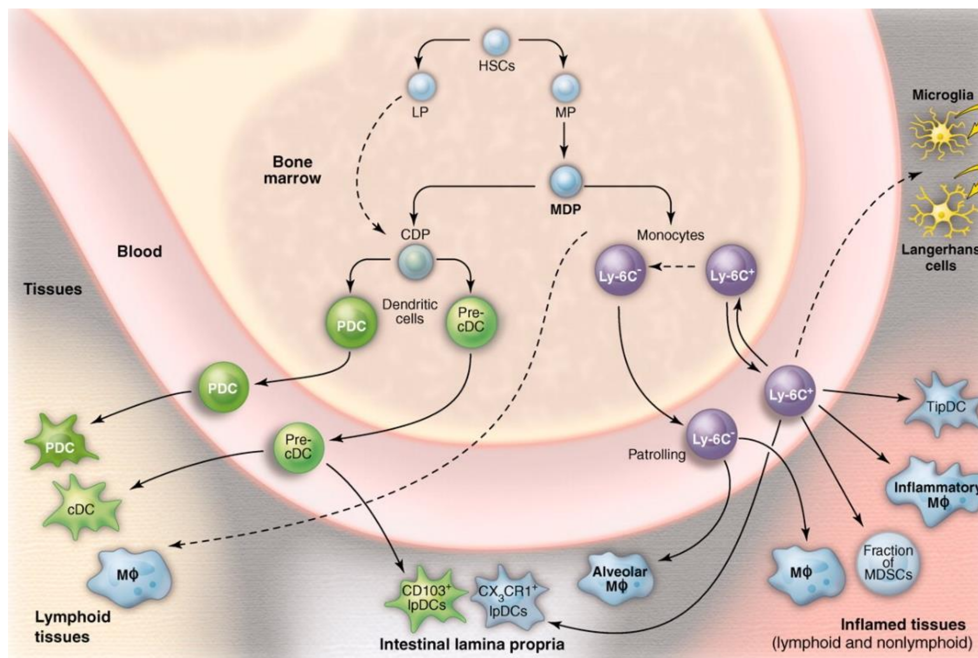

Figure 1.1. Differentiation of the various DC subsets from bone marrow. In the bone marrow, hematopoietic stem cells (HSCs) produce myeloid (MP) and lymphoid (LP) committed precursors. MPs give rise to monocyte, macrophage, and DC precursors (MDPs). MDPs give rise to monocytes and common DC precursors (CDPs). CDPs give rise to preclassical dendritic cells (pre-cDCs) and plasmacytoid dendritic cells (PDCs). Pre-cDCs circulate in blood and enter the tissue, where they give rise to cDCs. During inflammation, Ly-6C$^+$ monocytes give rise to monocyte-derived DCs, such as tumor necrosis factor (TNF) and inducible nitric oxide synthase (iNOS)–producing dendritic cells (TipDCs). They are also

suspected to contribute to Langerhans cells (170). Reprinted with the permission from the author and AAAS publisher.

1.2.2 Functional role of DC in keeping the immune balance

DC are the sentinels of the immune system. They play an essential role in immunosurveillance by searching their environment to augment the chance in capturing antigens. As previously described, DC are armed to respond rapidly and appropriately to any given intrinsic and extrinsic stimulus. Before we go deeper into how DC keep the balance between immunity and tolerance, we describe two different functional types of DC, namely the migratory DC and the tissue-resident DC. Migratory DC are found in peripheral tissues, such as the skin, and continuously sample the environment. As indicated by the name, these DC take up antigens and start migrating towards local lymph nodes, where they present these antigens to T cells. Tissue-resident DC in contrast do not circulate. They remain at their location in the tissue and consequently can only process antigens from the tissue they reside in. These two different DC types are not two separate entities but cross-talk exists between them. Migratory DC are capable to transfer antigens to lymphoid tissue-resident DC. In this way the limited access to antigens for tissue-resident DC is overcome (157, 171).

Besides antigen-uptake and presentation, the main feature of a DC is providing a link towards the adaptive immune system. More specifically, DC play an essential role in T cell polarization. Not only are they capable of inducing immune responses against foreign substances, they are also involved in keeping tolerance to self-antigens. In the thymus, T cells undergo negative selection. In this process, T cells that bind with high affinity to self-antigens presented by follicular DC are deleted via apoptosis. In this way, DC stop the majority of autoreactive T cells of being released to the periphery ("central tolerance"). Still, not all self-peptides of the human body are expressed in the thymus whereby some autoreactive T cells escape negative selection and reach the periphery. Some of these autoreactive T cells never come in contact with their antigen or cannot reach their antigens within immunologically privileged site such as the eye and CNS. If an autoreactive T cell can enter a tissue where its antigens are available they are controlled by a mechanism called peripheral tolerance (172). Immature DC continuously monitor peripheral tissues and capture antigens. Once the antigen is taken up, it is cleaved into peptides and expressed on the cell surface in a MHC-peptide complex. This

complex is subsequently presented to naive T cells and in the steady-state induces peripheral tolerance towards the self-antigen in question. In the steady-state, immature DC present antigens without proper co-stimulation to naïve T cells, such as CD80/86-CD28 ligation and the production of T cell polarizing cytokines. The absence of these signals leads to autoreactive T cell deletion, anergy or the induction of regulatory T (Treg) cells. When external stimuli are present such as viruses or bacteria, the immature DC is stimulated and shifts from tolerogenic to immunogenic. During activation DC upregulate co-stimulatory molecules and produce cytokines thereby providing secondary and tertiary signals leading to full activation of T cells and differentiation towards effector cells (149, 152, 173-175). To summarize, DC are not only essential components for mounting immune responses against invading pathogens, but also for inducing tolerance to self or regulating ongoing immune responses to prevent uncontrolled tissue damage and induction of an autoimmunity. As is clear from the different DC subtypes and functions, the outcome of an encounter between T cells and DC depends on the local environment and activation state of the DC.

1.2.3 Evidence for the involvement of DC in MS pathogenesis

Although research in MS is mainly focused on the role of the adaptive immune system, numerous studies suggest an important role for the innate immune system in the initiation and progression of MS. DC are pivotal in influencing and activating effector B and T cells. This interaction is bi-directional because the effector cells further activate DC via cytokines and activation markers. It is suggested that changes in the DC compartment can lead to the initiation, perpetuation or progression of an autoimmune disease such as MS.

Several studies indicate DC disturbances in autoimmune diseases including MS (176-181). In the animal model of MS, DC accumulate in the CNS during inflammation. These DC subsequently activate encephalitogenic T cells indicating that DC play an essential role in the EAE pathogenesis (179, 182, 183). Recent studies challenge these findings by showing that DC are dispensable for the initiation and primal activation of encephalitogenic T cells in EAE and are only important in the chronic progression phase by supporting the ongoing inflammation (184, 185). Also these new observations are under debate (186). In these studies CD11c-diphteria toxin receptor (DTR) mice were used leading to the ablation of $CD11c^{+}$ DC in the presence of diphtheria toxin. First, one needs to

consider that the efficiency of this model is not 100 percent. After deletion still 10-20% of DC remain in the dermis and lymph nodes. This percentage is sufficient to prime T cells (186). Second, as described above, the DC compartment is very heterogeneous. Not all of the subtypes express CD11c, so when interpreting studies like this, we have to keep in mind that other DC subtypes can take over the role of activating encephalitogenic T cells thereby initiating MS.
Altogether we can conclude from the multitude of studies that DC play an important role in shaping the immune response in MS by activating and attracting other immune cell as well as participating in the uptake of myelin and associated epitope spreading.

1.2.4 DC-based therapy

As DC determine the profile of immune responses, they form interesting immune modulation targets. DC were first exploited in the context of cancer research owning to their immune stimulatory potential (187). These studies aimed to boost a specific immune response against tumor cells. In light of its role in immunity and tolerance, the therapeutic potential of DC stretches far beyond enhancing immunity and can also be used to induce tolerance in transplantation, allergy or autoimmune disease. First clinical trials are already underway which exploit DC to treat allergic asthma and type 1 diabetes. However several general parameters must be satisfied and optimized before a DC therapy can be approved to treat patients.
The most important parameter in this approach is the generation of tolerogenic DC (tDC) that remain stable in vivo. In vivo stimuli can undermine the efficacy of tDC therapy as tDC may switch back to stimulate immune reactions when the appropriate activation and maturation signals are present. To generate stable tDC several developmental and functional stages of DC can be targeted. As is summarized in Figure 1.2, DC intervention with drugs can be envisaged at different levels: either during differentiation, antigen uptake, maturation or migration or in the effector stage (188, 189). Several stimuli exist that demonstrated to have tolerizing abilities on DC, such as vitamin D and IL-10 (190-192). Moreover there is an abundancy of studies testing new drugs to develop a tDC vaccine in the animal model of MS. An overview of these drugs and their effect

are given in Table 1.1. Unfortunately to date, there is no consensus concerning the stimuli that produce DC locked in a permanent tolerogenic state.

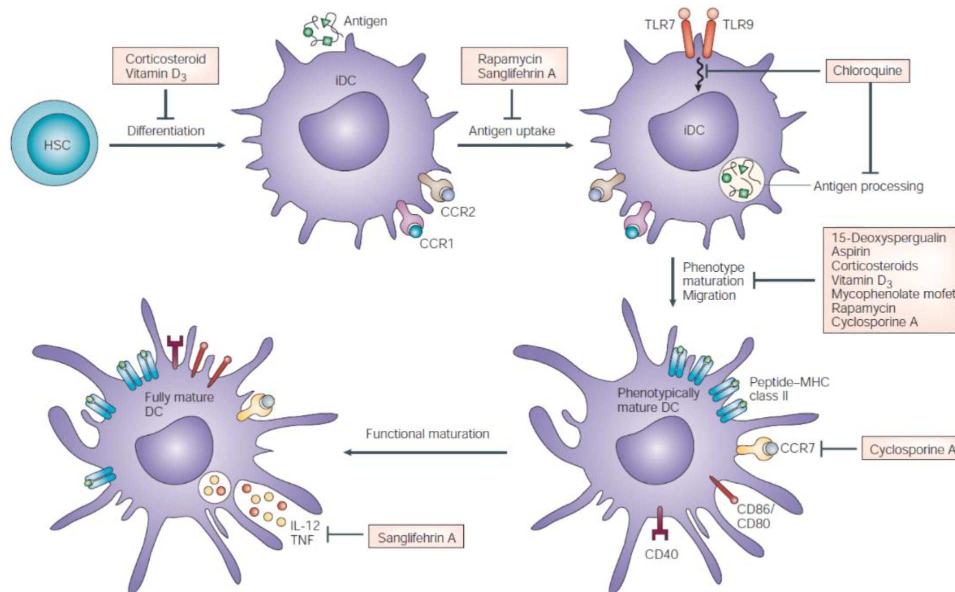

Figure 1.2. Possible mechanism to target dendritic cells and shift DC towards tolerogenic properties. Inhibitory effects can take place on DC differentiation, maturation and function. For example, corticosteroids and vitmanin D_3 suppress DC differentiation and maturation whereas rapamycin suppresses antigen uptake and maturation. This illustrates that therapeutic intervention in DC are possible on several levels. Combination of drugs that suppress different DC phases can lead to a better and stable DC with tolerogenic characteristics. For example, sanglifehrin A and vitamin D_3. In this way not only differentiation and maturation are blocked, but even the production of bio-active IL-12 and TNFα. Reprinted with the permission from the author and publisher (189). HSC = hematopoietic stem cell, iDC = immature DC, TLR = Toll-like receptor.

Table 1.1. Overview of the effects of tolerogenic DC therapies in EAE, the animal model of MS.

Tolerogenic stimulus	Category	EAE model	Injection scheme	Treatment effect	Ref.
TNF-α	Cytokine	MOG EAE	Repetitive prophylactic injections of TNF-DC	- Prevents EAE - Induction of IL-10 producing T cells	(193)
Mitomycin C	Chemo-therapeutic	MBP EAE	EAE induction with MBP loaded DC +/- Mitomycin C	- Prevents EAE - Modulates expression of apoptotic and immunoregulatory genes in DC leading to inhibition of T cells	(194)
MBP	Myelin component	MBP EAE	Prophylactic injection with adherent MBP-loaded DC	- Prevents EAE - Induces tolerance by IL-10 production	(195, 196)
Andrographolide Rosiglitazone	NF-κB inhibitor	MOG EAE	Profylactic injection with MOG loaded NF-κB inhibitor treated DC	- Prevents EAE - Blocking DC maturation leading to reduced capacity to activate T cells and generated regulatory T cells	(197)
Galectin-1	Glycan-binding protein	MOG EAE	Therapeutic injection with MOG loaded Galectin-1 treated DC	- Halts autoimmune inflammation - Roll for IL-27 and IL-10	(198)

1.3 Epigenetics and MS

Recent evidence suggests epigenetic changes provide the missing link between external exposures and genetic predisposition. In the following sections a detailed description of epigenetic mechanisms and their involvement in MS are given.

1.3.1 Epigenetic mechanisms

Epigenetics is the study of mechanisms that alter gene expression without altering the DNA sequence itself and include DNA methylations, histone modifications and microRNA (miRNA)-associated posttranscriptional gene silencing. Epigenetic changes are inherited and retained during cell divisions. However epigenetic changes are reversible and highly sensitive to environmental effects (199).

1.3.1.1 DNA methylation

DNA methylation is the process where methyl groups are added to the carbon-5 of cytosine residues in DNA. The addition of methyl groups is done by several methyltransferases (DNMT). For example, DNMT1 is an enzyme involved in the maintenance of DNA methylation patterns during DNA replication. DNMT3a and DMNT3b are responsible for *de novo* methylations in nuclear and mitochondrial DNA. In mammals CpG sites are the predominant place where DNA methylations take place. As a consequence of methylations of so-called CpG islands in promotor regions, the expression of the associated gene is repressed. As CpG island methylation changes the chromatine structure in the promotor region, it modifies interactions between the promotor and its transcription factors, thereby silencing the specific gene (199-201).

1.3.1.2 Histone modification

Histones are octamers build out of 2 copies of each histone protein, namely H2A, H2B, H3 and H4. These histones interact with the DNA to package the DNA into chromatin. The tightly packaging of DNA is needed because the nucleus cannot contain randomly distributed DNA. When we look at the packed form of DNA we can distinguish 2 different forms, euchromatin and heterochromatin. In euchromatin the DNA is lightly packed and genes undergo active transcription. Heterochromatin on the other hand is a tightly packed form of DNA and hence associated with gene repression. Nucleosomal histones have tails that are rich in lysines and arginines. These amino acids can be modified by acetylation,

methylation, phosphorylation, ubiquitination and citrullination. Acetylation is one of the most important regulatory mechanisms of gene expression and is done by histone acetyltransferases (HAT). Histone acetylation contributes to the upregulation of the transcription of the gene in question. The opposite is true for deacetylation of histones by histone deacetylases (HDAC). In general, histone modifications are influenced by external stimuli. Moreover, histone modifications work together with DNA methylation in silencing certain genes. DNA methylation intervenes with the direct binding of transcription factors to the promotor region of the gene, whereas HDAC deacetylates the histones leading to compacting the DNA. In this way, the specific gene is efficiently silenced (199-201).

1.3.1.3 miRNA-associated gene silencing

MiRNA are single-stranded, noncoding RNA of about 22 nucleotides long that can suppress translation by binding to complementary target mRNA. miRNA are formed when primary miRNA transcripts are processed in the nucleus by the RNase III Drosha and subsequently in the cytoplasm by Dicer. One of the strands undergoes degradation, whereas the other strand is incorporated into the RNA-induced silencing complex (RISC). This complex binds to the 3'-untranslated region of complementary mRNA and the mRNA is degraded. Via this mechanism, mRNAs are destroyed and translation of the target mRNA is inhibited. The process of miRNA-associated gene silencing is involved in many important biological processes such as development, differentiation, proliferation and apoptosis (199, 200).

1.3.2 Evidence for the role of epigenetic mechanisms in MS

As described earlier, MS is a complex and heterogeneous disease that is assumed to develop by an interplay between genetic and environmental factors. Genetics cannot fully explain the development of MS as the disease concordance of monozygotic twins is only moderate. Studies have highlighted the importance of environmental factors that alongside genetic susceptibility can explain the increased risk in developing MS. Many environmental factors such as exposure to tobacco smoke, infectious agents, ultraviolet light/vitamin D and chemical compounds can modify gene expression through epigenetic mechanisms. Epigenetic changes lead to a modulation of gene expression associated with immune function. For these reasons, epigenetics can be seen as one of the missing

links that connect genetics and environmental effects in the development of MS (202).

1.3.2.1 Epigenetics as a diagnostic tool

Recent studies in MS aimed to use epigenetics as biomarkers for the diagnosis of MS. Liggett and colleagues compared methylation patterns in the DNA isolated from cell-free plasma between RRMS patients and healthy controls. They observed differences in methylation patterns between the 2 groups and were able to distinguish MS patients from healthy controls with a sensitivity of >75% and a specificity of >90%. Moreover, based on methylation patterns, they could separate RRMS patients in patients in remission or during relapse (203). Another study found 165 miRNAs that are different between MS patients and healthy controls (204). An overview of studies that exploit epigenetic changes for the diagnosis of MS are found in Table 1.2. The use of a biomarker panel of epigenetic changes is promising. In this way, it could be possible to diagnose MS patients much earlier and more specifically, although larger confirmatory studies are needed. Epigenetic biomarkers may also provide information on which patients will develop additional lesions or which have a tendency to progress. Moreover, the effect of immunomodulatory drugs on epigenetic biomarkers can be monitored and help predict which patients would benefit from the treatment. Of course, it is too early to make such promising prospectives since research in epigenetics and their use as a biomarker is still in its infancy (205).

Table 1.2. Possible epigenetic biomarkers in MS. Adapted with permission from the author and publisher (205).

Possible biomarker	Sensitivity/ Specificity	Study cohort	Tissue	Ref
Diagnostic biomarkers				
DNA methylation patterns	Sensitivity: >75% Specificity: >90%	60 RRMS, 30 HC	Cell-free blood plasma	(203)
Has-miR-145	Sensitivity: 89.5% Specificity: 90%	20 RRMS, 19 HC	Whole blood	(204)
Panel of has-miR-155, hsa-miR-146a and has-miR-142-3p	Sensitivity: 77.8% Specificity: 88%	36 RRMS, 32 HC	PBMC	(206)
Biomarkers associated with disease activity and treatment response				
DNA methylation patterns	Sensitivity: 70.8% Specificity: 71.2%	30 RRMS in remission vs. 29 RRMS during relapse	Cell-free blood plasma	(203)
Has-miR-18b and has-miR-599 (relapse) and has-miR-96 (remission)	N.D.	9 RRMS in remission vs. 4 RRMS during relapse vs. 8 HC	PBMC	(207)
has-miR-146a (glatiramer) and has-miR-142-3p (INFβ)	N.D.	20 glatiramer acetated treated RRMS vs. 18 IFNβ treated RRMS vs. 36 untreated RRMS vs. 32 HC	PBMC	(206)

Abbreviations: PBMC = Peripheral blood mononuclear cells, RRMS = relapsing remitting MS, HC = Healthy control, IFNβ = interferon β, N.D. = Not determined.

1.3.2.2 Epigenetics in MS pathology

Studies suggest that epigenetic changes are involved in the pathology of MS by modulating immune functions. A possible mechanism of a direct role of epigenetics is the hypomethylation of the peptide arginine deiminase 2 (PAD2). This enzyme is involved in citrullination of MBP and overexpression of PAD2 leads to a higher level of citrullinated MBP. This citrullination of MBP causes a positive charge which

interferes with the interaction between MBP and the lipid bilayer. Consequently, citrullination can lead to a loss of myelin stability in MS (208). Moreover, oligodendrocytes in chronic MS lesions showed enhanced acetylations of histone H3. This specific acetylation in oligodendrocytes is associated with impaired differentiation and could lead to impaired remyelination (209). Further evidence comes from the observation that epigenetic changes are needed to establish polarization towards certain T cell lineages (210, 211). A study by Janson et al. showed that hypomethylation of the IL17a promoter region results in increased frequencies of T cells that differentiate towards Th17 cells (212). Furthermore, a conserved CpG-rich island in the Foxp3 promoter region, the Treg-specific demethylated region (TSDR), is under epigenetic control. Hypomethylation in this region leads to a stable and strong expression of Foxp3 (213). This continuous expression of Foxp3 is essential for the immunosuppressive function of regulatory T cells and in MS it is proven that regulatory T cells are indeed dysfunctional. As a consequence autoreactive T cells are able to resist their immunosuppressive effects (83-86). Lastly, miRNA are dysregulated and associated with changes in immune function in MS patients. In MS patients, miR-17 and miR-20a are downregulated and these miRNA are associated with inhibition of T cell activation (214). Furthermore, miR-155, miR-34a and miR-326 are upregulated in active MS lesions. Normally these miRNA target CD47. CD47 inhibits phagocytosis of macrophages, meaning that an upregulation of these specific miRNA leads to macrophages with an increased phagocytic activity (215). miR-155 is also involved in T cell differentiation and is upregulated in response to inflammatory cytokines and TLR signaling. EAE animals that are deficient in this miRNA do not show development of EAE and this resistance is suggested to result from reduced Th17 generation (199). The transcription factor C-ets-1 inhibits the differentiation of naïve T cells towards Th17 cells and is under control of miR-326. Du et al. found that the expression of C-ets-1 was lower in RRMS compared to healthy controls (216).

To conclude, there is mounting evidence that epigenetic mechanisms are involved in the pathology of MS. Not only do epigenetic changes contribute to a dysregulated immune function (Th17/Treg), it also intervenes with myelin itself (PAD2). Although current studies have gained more insights in the exact role of epigenetics in inflammatory and complex diseases such as MS, more studies are needed to fully unravel the role of epigenetics in inflammation.

1.3.3 Exploiting epigenetic mechanisms to treat MS

The increasing evidence lend by epigenetic changes in inflammation lead to new therapeutic strategies in the treatment of autoimmune diseases. Hypomethylating agents have proven successful in treatment of cancer. For example decitabine (DAC) has been widely studied in cancer research and is approved for the treatment of myelodysplasic syndrome (MDS) (217-221). It was shown to improve outcomes of patients with MDS (217-219). Hypomethylation in this case is associated with reactivation of multiple genes, including tumor-suppressor genes and induction of cell death (222, 223). Besides the use of hypomethylating agents in cancer research, lower doses can also be used to modulate the immune function in immunological disease. In this context it was demonstrated that trichostatin A, histone deacetylase inhibitor, ensured neuronal survival and countered pro-inflammatory responses thereby ameliorating clincical symptoms in EAE. The neuroprotection achieved by trichostatin A is likely due to promotion of antioxidants, anti-excitotoxicity, hormonal and pro-neuronal growth responses, inhibition of caspase activity and reduction of mRNA that induces Th1 responses (224). Another histone deacetylase inhibitor, called vorinostat, was able to suppress DC functions resulting in a less severe EAE course. Ge and colleagues showed that costimulatory molecules on DC were reduced together with a decreased production in Th1 and Th17 polarizing cytokines. Furthermore, vorinostat also directly suppressed pathogenic Th1 and Th17 cells in EAE mice (225). Interfering with DNA methylation patterns can also be beneficial in inflammation. It was demonstrated that DAC regulates Foxp3 expression in T cells and treatment of mice with DAC inhibited the occurrence of diabetes (226). Lastly, miRNA-associated gene silencing also shows promise for treating autoimmune diseases. For example, silencing miR-155 by injecting an antisense oligonucleotide was able to attenuate EAE symptoms when administered before EAE induction (199).

To summarize, epigenetics is a new and promising field for the treatment of autoimmune diseases. Further research in epigenetic changes in MS patients and may lead to new diagnostic or prognostic markers and targets for therapy.

1.4 Aims of the study

Immune regulation is an important mechanism to establish homeostasis in the immune system. Via this way, the immune system can maintain tolerance towards the organism's own healthy tissue and detect and mount an appropriate response against invading pathogens. A disturbance in the immune regulatory mechanisms can lead to the induction or perpetuation of immunological disease.

A central role in immune regulation is reserved for DC. DC belong to innate immunity and are widely known as professional antigen-presenting cells. They are able to influence both immunity and tolerance. Not only are they the main inducers of primary T cell responses, DC also determine the profile of those responses depending on the co-stimulation they provide. Because of their unique capacity to shape immune responses, DC are thought to play a pivotal role in the immunopathogenesis of several autoimmune disorders, including MS. A second factor that affects immune regulation is epigenetic modification. Studies have shown that DNA methylation and histone modifications can regulate the expression of genes associated with the immune system, thereby modifying the development of the innate and adaptive immune responses. In this study we evaluated whether DC targeting or epigenetic modulation is able to restore self tolerance in the context of multiple sclerosis. The following aims were set forward to approach this general aim:

<u>Aim I: Characterization of the DC compartment in MS patients</u>

MS is generally accepted to develop in genetically predisposed individuals as a result of breakdown of tolerance to self. Whereas immune dysregulation in MS has been documented extensively at the level of the adaptive immune system, recent studies have suggested that DC, which are innate immune cells, may also play a role in the pathogenesis of this disease. In **chapter 2**, we further elaborate on previous studies by determining if circulating DC subsets undergo changes in phenotypical, functional and migratory profiles in an extensive cohort of MS patients during different disease stages. Moreover, we investigate for the first time whether the DC compartment is influenced by MS-associated genetic risk alleles in immunological genes such as HLA-DRB1*1501 and IL-7Rα. Peripheral blood of 110 MS patients was analysed for the following DC parameters: frequency of mDC and pDC, costimulatory and migratory phenotype and capacity, TLR responsiveness and association with genetic risk factors of MS.

<u>Aim II: Generation of tolerogenic DC by the histone deacetylase inhibitor SAHA to restore the immune tolerance in EAE</u>

Given that DC are altered in MS and have the ability to shape the immune response by polarizing T cells into different effector cell types, there is significant interest to use DC to limit immunopathogenic responses. In **chapter 3**, we examine whether the histone deacetylase inhibitor SAHA is able to generate tolerogenic DC that counteract autoimmune responses in an animal model of MS. SAHA (vorinostat) was already shown to induce a less severe EAE course when directly administered *in vivo*. SAHA was able to suppress costimulatory molecules on DC *in vivo* leading to reduced Th1 and Th17 polarizing cytokines. Since SAHA likely affects other genes and cell types, possibly resulting in unwanted side effects, we chose to adapt the therapeutic SAHA mediated approach by generating tolerogenic DC in vitro and then apply them *in vivo*. First the optimal protocol was identified to induce stable tolerogenic DC *in vitro*. This is accomplished by generating DC out of mouse bone marrow under different SAHA conditions and subsequently determining a panel of essential DC characteristics: more specifically antigen uptake and presentation, costimulatory capacity and cytokine production. We further identify the signaling induced by SAHA on DC. Next, SAHA-generated DC were evaluated for their capacity to induce tolerance in myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$-induced chronic EAE. To address this, we injected EAE mice with PBS, MOG-pulsed or MOG-pulsed SAHA-generated DC and monitored for disease severity and pathology.

<u>Aim III: Targeting regulatory mechanisms *in vivo* by DNA methylation inhibition to counteract EAE</u>

Instead of generating tolerogenic DC with DAC in vitro as was done in chapter 2 with SAHA, we next aim to counteract the autoimmune response by intervening directly with DNA methylation in vivo to induce regulatory mechanisms that are not merely aimed to induce DC tolerisation. In chapter 4 we study the effect of DAC treatment on the disease course of chronic EAE. EAE mice are intraperitoneally treated on a daily base with DAC (0.15 mg/kg) and evaluated for disease severity and CNS immune cell infiltration. Moreover changes with regard to the T cell compartment in primary and secondary lymphoid organs are monitored and underlying mechanisms identified.

2

Circulating dendritic cells of multiple sclerosis patients are proinflammatory and their frequency is correlated with MS-associated genetic risk factors

Based on:

Circulating dendritic cells of multiple sclerosis patients are proinflammatory and their frequency is correlated with MS-associated genetic risk factors

Kristof Thewissen[1]*, Amber H. Nuyts[2]*, Nathalie Deckx[2], Bart Van Wijmeersch[1,3,4], Guy Nagels[5], Marie D'hooghe[5], Barbara Willekens[6], Patrick Cras[6], Bert O. Eijnde[1,4], Herman Goossens[2], Viggo F.I. Van Tendeloo[2], Piet Stinissen[1], Zwi N. Berneman[2], Niels Hellings[1]**, Nathalie Cools[2]**
Mult Scler. 2014 Apr;20(5):548-57

[1] Biomedical Research Institute, Hasselt University, Belgium
[2] Vaccine & Infectious Disease Institute (Vaxinfectio), University of Antwerp, Belgium
[3] Revalidation & MS Center, Belgium
[4] Dpt. Healthcare, REVAL Rehabilitation Research Center, Belgium
[5] Department of Neurology, National MS Center, Belgium
[6] Division of Neurology, Antwerp University Hospital, Belgium
* Both authors contributed equally to this work.
** Both senior authors contributed equally to this work.

ABSTRACT

The role of the adaptive immune system and more specifically T cells in the pathogenesis of multiple sclerosis (MS) have been studied extensively. Since emerging evidence suggests that the innate immune system contributes to the pathogenesis of MS, we hypothesized that alterations in frequency and function of dendritic cells (DC) contribute to autoimmunity. An ex vivo analysis of conventional (cDC) and plasmacytoid DC (pDC) in the peripheral blood of MS patients (n=110; 2 CIS, 50 RRMS, 58 CPMS) and age- and gender-matched healthy controls (n=112) was performed. Circulating pDC were significantly decreased in patients with chronic progressive MS (CPMS) compared to relapsing-remitting (RR)MS and healthy controls. While no differences in cDC frequency were found between the different study groups, HLA-DRB1*1501^{+} MS patients and patients not carrying the protective IL-7Rα haplotype 2 have reduced frequencies of circulating cDC and pDC respectively. MS-derived DC showed enhanced IL-12p70 production upon TLR ligation and had an increased expression of the migratory molecules CCR5 and CCR7 as well as an enhanced in vitro chemotaxis.

Altogether, our data provides evidence to a dual role of circulating DC in the pathogenesis of MS. cDC are in a more pro-inflammatory state in MS and drive immune responses that lead to damage in the CNS. In contrast, pDC are known to regulate immune responses, but in MS patients we suggest that these cells have a diminished regulatory capacity. The different roles of DC subtypes in an immune response can open new therapeutic strategies, like for example boosting regulatory pDC in counteracting an autoimmune reaction.

2.1 Introduction

Multiple sclerosis (MS) is generally accepted to develop in genetically predisposed individuals as a result of breakdown of tolerance to self. Whereas immune dysregulation in MS has been documented extensively at the level of the adaptive immune system (85, 87, 227), recent studies have suggested that DC, which are innate immune cells, may also play a role in the pathogenesis of this disease. Due to their specialized antigen-presenting capacity, DC provide an important link between the innate and the adaptive immune system. They play an important role in polarizing the T cell response, regulating the balance between immunity and tolerance (174, 228, 229). Within the DC network, several subsets orchestrate specific roles in immunosurveillance, migration and antigen presentation (230). In human blood, based on their progenitor origin, two major subsets are distinguished: myeloid or conventional DC (cDC) and plasmacytoid DC (pDC) (231). Differences in expression of Toll-like receptor (TLR) (232), cytokine receptors and cytokines (176, 177, 230, 233), as well as a difference in migratory potential point to a different function in induction and regulation of the immune response by both DC subtypes. Because of their unique capacity to shape immune responses (234), DC are thought to play a pivotal role in the immunopathogenesis of several auto-immune disorders (233, 235), including MS.
In general, data of human DC studies demonstrate that both DC subsets in the peripheral blood of MS patients are altered and more prone to drive a proinflammatory T cell response (176-178). However, some studies show conflicting results on the functionality of circulating DC in MS patients (178, 236). The first aim of the present study was to re-examine circulating DC subsets in a large cohort of MS patients taking into account the influence of medication as well as the clinical phase of MS patients. The most common clinical course is relapsing-remitting MS (RRMS) usually followed by a chronic progressive phase of the disease (CPMS). Factors associated with this change remain elusive. Karni et al. proposed that major changes in the peripheral immune compartment of MS patients could trigger transition of the disease to the progressive state (176). Therefore it is important to obtain a full overview of the distinct phenotypes of circulating DC during various stages of the disease.

E*x vivo* analyses of the frequency and expression of costimulatory (CD80, CD86) and migratory (CD62L, CCR5 and CCR7) molecules of DC subsets were performed and the *in vitro* responsiveness to TLR challenge was studied.
Genetic predisposition enhances susceptibility to MS with the human leukocyte antigen (HLA) class II genes accounting for 14-50% of the genetic burden. HLA-DRB1*1501 carriers have a 3-4 times higher relative risk of developing MS (20, 23). One of the first non-HLA gene loci linked to MS susceptibility is the gene for the IL-7 receptor α-chain (IL-7Rα) (237, 238). IL-7 signalling is essential for central T cell development and T cell homeostasis. (239, 240). Information regarding the effect of susceptibility genes on DC remains elusive. In the second part of this chapter, MS patients were genotyped for HLA-DRB1*1501 and IL-7Rα variants to examine whether these MS-associated genetic risk factors affect the DC compartment. Overall, this chapter aimed at providing more insights into the contribution of DC in MS and to determine whether MS-associated genetic risk factors influence DC subtypes.

2.2 Materials and methods

2.2.1 Study population

A total of 110 MS patients (43 male and 67 female), diagnosed according to McDonald criteria (11), were recruited by the Department of Neurology from the National MS Center (Melsbroek, Belgium), the MS Center Overpelt (Overpelt, Belgium) and the Division of Neurology from the Antwerp University Hospital (Edegem, Belgium). Patient characteristics and medication taken at time of sample collection is depicted in table 2.1. 112 age- and gender-matched healthy controls were recruited. In MS patients and healthy controls the mean age was 49 years (range 19 – 75 years), and 45 years (range 23 – 77 years) respectively. Furthermore, we strived for an equal distribution of men and women in all study groups, i.e. 43/67 male female ratio in MS patients as well as 44/68 male female ratio for healthy controls. The median expanded disability status scale (EDSS) score of RRMS patients was 2.5 (range 0 – 7.5) and of CPMS patients was 6 (range 1.5 – 8.5). All subjects gave informed consent in accordance with the declaration of Helsinki and the protocol was approved by the local Ethics Committees of the respective MS centres as well as the local Ethics Committee of the Antwerp University Hospital and Hasselt University.

Table 2.1. Clinical details of the patients recruited into the study.

	MS TYPE	EDSS	MEDICATION	AGE	GENDER		MS TYPE	EDSS	MEDICATION	AGE	GENDER
UPN001	CPMS	6.5	None	44	Male	UPN056	RRMS	3	IFN-β	56	Male
UPN002	RRMS	6.5	None	42	Male	UPN057	RRMS	5.5	Other	58	Female
UPN003	CPMS	5.5	None	52	Female	UPN058	CPMS	4	IFN-β	52	Female
UPN004	RRMS	5.5	Other	36	Female	UPN059	RRMS	2	None	51	Female
UPN005	RRMS	3.5	Other	50	Female	UPN060	RRMS	2	Other	60	Female
UPN006	RRMS	7.5	IFN-β	43	Male	UPN061	RRMS	6	Other	60	Male
UPN007	RRMS	5	IFN-β	36	Female	UPN062	RRMS	2.5	IFN-β	47	Female
UPN008	RRMS	5.5	IFN-β	54	Female	UPN063	RRMS	2	IFN-β	52	Female
UPN009	CPMS	7	Other	45	Male	UPN064	CPMS	2.5	IFN-β	65	Male
UPN010	CPMS	8	None	43	Male	UPN065	RRMS	1.5	IFN-β	50	Female
UPN011	CPMS	7	IFN-β	57	Female	UPN066	RRMS	2.5	None	52	Female
UPN012	CPMS	7	Other	52	Male	UPN067	RRMS	3	IFN-β	32	Male
UPN013	CPMS	7.5	None	75	Male	UPN068	CPMS	6	IFN-β	55	Female
UPN014	CPMS	8.5	None	55	Male	UPN069	CPMS	5.5	IFN-β	60	Male
UPN015	CPMS	6.5	IFN-β	50	Female	UPN070	RRMS	3	Other	41	Female
UPN016	CPMS	6	None	61	Female	UPN071	RRMS	2.5	None	19	Female
UPN017	CPMS	6.5	None	41	Male	UPN072	CPMS	6	None	19	Male
UPN018	CPMS	7	Other	42	Male	UPN073	CPMS	7.5	IFN-β	51	Female
UPN019	CPMS	8.5	None	63	Male	UPN074	RRMS	0	None	39	Female
UPN020	RRMS	2	IFN-β	35	Male	UPN075	RRMS	2.5	None	51	Female
UPN021	RRMS	1.5	IFN-β	38	Female	UPN076	CPMS	5.5	None	63	Female
UPN022	RRMS	2.5	None	46	Female	UPN077	RRMS	2	None	44	Female
UPN023	CPMS	7	None	53	Male	UPN078	RRMS	4	IFN-β	56	Male
UPN024	CPMS	7.5	None	70	Female	UPN079	RRMS	2	IFN-β	57	Female
UPN025	RRMS	2	None	45	Female	UPN080	CPMS	6	IFN-β	53	Female
UPN026	CPMS	5.5	None	55	Female	UPN081	RRMS	1.5	IFN-β	47	Female
UPN027	CPMS	6	None	55	Female	UPN082	CPMS	2.5	None	53	Male
UPN028	CPMS	1.5	None	54	Male	UPN083	RRMS	2.5	Other	43	Female
UPN029	CPMS	8	IFN-β	46	Male	UPN084	RRMS	2	None	33	Female
UPN030	CPMS	7	None	50	Female	UPN085	CPMS	3.5	IFN-β	44	Female
UPN031	CPMS	8	None	56	Female	UPN086	CPMS	6.5	None	43	Female
UPN032	CPMS	8	None	61	Female	UPN087	RRMS	3	None	46	Female
UPN033	CPMS	8	None	54	Female	UPN088	CPMS	7	None	35	Male
UPN034	CPMS	9	None	31	Female	UPN089	CPMS	6.5	Other	62	Female
UPN035	CPMS	8	None	45	Female	UPN090	RRMS	2.5	IFN-β	34	Female
UPN036	CPMS	8	None	54	Male	UPN091	CPMS	3.0	Other	59	Female
UPN037	CPMS	6.5	None	51	Male	UPN092	CPMS	3.0	Other	55	Male
UPN038	CPMS	6	None	49	Female	UPN093	RRMS	4.0	IFN-β	40	Male
UPN039	CPMS	4	IFN-β	40	Male	UPN094	RRMS	2.5	IFN-β	47	Female
UPN040	RRMS	2	IFN-β	50	Male	UPN095	CPMS	6.0	None	49	Female
UPN041	CPMS	6	None	64	Male	UPN096	CPMS	4.5	Other	62	Male
UPN042	CPMS	3	None	58	Female	UPN097	CPMS	6.5	IFN-β	53	Female
UPN043	CPMS	4.5	None	49	Male	UPN098	RRMS	3.0	IFN-β	31	Male
UPN044	CPMS	2.5	IFN-β	52	Female	UPN099	RRMS	2.5	IFN-β	52	Male
UPN045	CPMS	3.5	None	59	Male	UPN100	CPMS	6.0	None	63	Male
UPN046	RRMS	1.5	Other	38	Female	UPN101	RRMS	2.0	IFN-β	52	Female
UPN047	RRMS	2.5	IFN-β	54	Male	UPN102	RRMS	2	IFN-β	54	Female
UPN048	RRMS	1.5	Other	27	Male	UPN103	RRMS	2	IFN-β	25	Female
UPN049	RRMS	1	Other	39	Female	UPN104	RRMS	NK	None	59	Male
UPN050	RRMS	2.5	Other	45	Female	UPN105	CPMS	3	IFN-β	48	Female
UPN051	CPMS	4	Other	45	Female	UPN106	RRMS	1,5	None	53	Female
UPN052	CPMS	4	IFN-β	29	Male	UPN107	RRMS	1,5	IFN-β	48	Female
UPN053	RRMS	2.5	IFN-β	59	Female	UPN108	CIS	0	None	26	Female
UPN054	RRMS	2.5	IFN-β	41	Female	UPN109	CPMS	NK	NK	NK	Male
UPN055	CPMS	4	Other	51	Female	UPN110	CIS	2,5	NK	63	Male

Abbreviations: MS: multiple sclerosis, RRMS: relapsing-remitting MS, CPMS: chronic progressive MS, UPN: unique patient number, EDSS: Expanded Disability Status Scale, IFN:

interferon. IFN-β therapy (i.e. Avonex®, Betaferon® or Rebif®) or other [i.e. co-polymer 1 (Copaxone®), cyclophosphamide (Endoxan®), natalizumab (Tysabri®)].

2.2.2 Isolation and stimulation of leukocytes

Prior to *ex vivo* staining, leukocytes were enriched by density gradient purification (Ficoll Pacque PLUS, GE Healthcare, Chalfont St. Giles, UK) or by red blood cell lysis (Miltenyi Biotec, Leiden, The Netherlands). In other experiments, DC were isolated using the EasySep pan-DC pre-enrichment kit (Stemcell Technologies, Grenoble, France).

To evaluate DC function, 1 mL of peripheral blood was stimulated for at least 16 hr with a TLR4 ligand, lipopolysaccharide (LPS, 2 μg/ml, Invivogen, Toulouse, France) and interferon (IFN)-γ (50 μg/mL, Immunotools, Friesoythe, Germany) or with a TLR7 ligand, imiquimod (IQ, 10 μg/ml, Invivogen) or left untreated as a control. After centrifugation, plasma was collected and stored at -20°C for future measurements of IL-12p70 and IFN-α production.

2.2.3 Flow cytometry

To characterize DC subsets, leukocytes were stained with the following mouse anti-human antibodies: anti-BDCA-1, anti-BDCA-2, anti-CD14, anti-CD19 and 'dead cell discriminator' or a control mixture, according to manufacturer's instructions (Human Blood Dendritic Cell Enumeration Kit, Miltenyi Biotec). Immunophenotyping of DC subsets was done by analysis of the expression of DC-related membrane markers with the following antibodies: anti-CD80 (BD Biosciences, Erembodegem, Belgium), anti-CD86 (BD Biosciences), anti-CD62L (eBioscience, Vienna, Austria), anti-CCR5 (BD Biosciences), anti-CCR7 (BD Biosciences) and anti-HLA-DR (BD Biosciences) antibodies. Non-reactive isotype-matched antibodies were used as controls. For analytical flow cytometry at least 1×10^6 mononuclear cells were analyzed using a Cyflow ML flow cytometer (Partec, Münster, Germany) or a FACSAria II (BD Biosciences). All results were analyzed using Kaluza software (Beckman Coulter, Suarlée, Belgium).

2.2.4 In vitro migration assay

In vitro migration assays were performed using 24-transwell (5.0-mm pore size) plates (Corning Costar, Amsterdam, The Netherlands). In brief, 2×10^5 DC were plated in the upper compartment of a 5 μM pore transwell support in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Life Technologies,

Ghent, Belgium). Culture medium supplemented with different chemokines (250 ng/ml) was added to the lower compartment. CCL19 and CCL21 (R&D systems, Abingdon, United Kingdom) were combined to test CCR7 migration and MIP-1β and RANTES (R&D systems) for CCR5 migration. No chemokines were added to the culture medium as a negative control. As a positive control, 2 x 10^5 DC were added directly to the lower compartment. After 180 min at 37°C in a humidified 5% CO_2 incubator, DC were harvested and absolute cell numbers were counted using flow cytometric analysis (FACSAria II, BD Biosciences) at a fixed flow rate during 2 min. Percentage of migrating DC was calculated using the following equation: $[(\text{counts per minute } (cpm)_{\text{chemokine-driven migration}} - cpm_{\text{negative control}})/cpm_{\text{positive control}}] \times 100$.

2.2.5 Cytokine ELISA

Thawed plasma samples were analyzed for the presence of IL-12p70 (eBioscience, Vienna, Austria) and IFN-α (PBL InterferonSource, Piscataway, New Jersey), using ELISA kits according to manufacturer's protocol.

2.2.6 HLA-DRB1*1501 and IL-7Rα genotyping

HLA-DRB1*1501 and IL-7Rα genotypes were determined from genomic DNA isolated from leukocytes of MS patients (table 2.2) and healthy controls as previously described (25, 241).

2.2.7 Statistics

For statistical analysis, results were first analyzed using a two–way ANOVA including MS type, medication and their mutual interaction as variables. In case no significant interaction could be demonstrated, the model was simplified excluding the variable. In case no significant influence of medication could be demonstrated by means of aforementioned statistical method, a one-way ANOVA was performed to test differences regarding MS type. In case significant differences between groups ($p < 0.05$) were found, further analyses using Tukey post-hoc test were performed. For completeness, when a significant difference using the Levene's test was used, the data were log-transformed. Correlations of DC frequencies with genetic risk factors were statistically tested for contingency with a Chi-square test. All data were calculated using SPSS statistics version 19

(IBM, Brussels, Belgium). Graphs were generated in GraphPad version 5 software (Prism, La Jolla, CA, USA).

Table 2.2. Clinical and genetic background of the patients recruited into the study.

	MS TYPE	EDSS	MEDICATION	AGE	GENDER	HLA-DRB1*1501	IL-7Rα
UPN074	RRMS	0	None	39	Female	Negative	Hap2
UPN075	RRMS	2.5	None	51	Female	Negative	Hap2
UPN077	RRMS	2	None	44	Female	Negative	Hap2
UPN080	CPMS	6	IFN-β	53	Female	Positive	Non hap2
UPN081	RRMS	1.5	IFN-β	47	Female	Positive	Non hap2
UPN082	CPMS	2.5	None	53	Male	Positive	Non hap2
UPN083	RRMS	2.5	Other	43	Female	Negative	Hap2
UPN084	RRMS	2	None	33	Female	Positive	Non hap2
UPN085	CPMS	3.5	IFN-β	44	Female	Positive	Hap2
UPN086	CPMS	6.5	None	43	Female	Negative	Hap2
UPN087	RRMS	3	None	46	Female	Negative	Non hap2
UPN088	CPMS	7	None	35	Male	Negative	Non hap2
UPN089	CPMS	6.5	Other	62	Female	Negative	Non hap2
UPN090	RRMS	2.5	IFN-β	34	Female	Positive	Non hap2
UPN091	CPMS	3.0	Other	59	Female	Positive	Non hap2
UPN092	CPMS	3.0	Other	55	Male	Negative	Hap2
UPN093	RRMS	4.0	IFN-β	40	Male	Negative	Hap2
UPN094	RRMS	2.5	IFN-β	47	Female	Positive	Non hap2
UPN095	CPMS	6.0	None	49	Female	Negative	Non hap2
UPN096	CPMS	4.5	Other	62	Male	Negative	Non hap2
UPN097	CPMS	6.5	IFN-β	53	Female	Negative	Non hap2
UPN098	RRMS	3.0	IFN-β	31	Male	Positive	Non hap2
UPN099	RRMS	2.5	IFN-β	52	Male	Positive	Non hap2
UPN100	CPMS	6.0	None	63	Male	Negative	Hap2
UPN101	RRMS	2.0	IFN-β	52	Female	Positive	Non hap2
UPN109	CPMS	NK	NK	NK	Male	Negative	Hap2
UPN110	CIS	2,5	NK	63	Male	Negative	Hap2

Abbreviations: MS: multiple sclerosis, RRMS: relapsing-remitting MS, CPMS: chronic progressive MS, UPN: unique patient number, EDSS: Expanded Disability Status Scale, IFN: interferon. IFN-β therapy (i.e. Avonex®, Betaferon® or Rebif®) or other [i.e. co-polymer 1 (Copaxone®), cyclophosphamide (Endoxan®), natalizumab (Tysabri®)].

2.3 Results

2.3.1 pDC, but not cDC frequency is decreased in the peripheral blood of CPMS patients as compared to RRMS patients and healthy controls

We first investigated the relative proportion of circulating DC subsets, more specifically cDC and pDC, in healthy controls (n=78) and patients with RRMS (n=29) and CPMS (n=43). Differences in mean percentage of cDC in the mononuclear fraction were not significant between the MS cohorts compared to healthy controls (Figure 2.1a). The mean percentage of pDC was significantly decreased in patients with CPMS (0.23 ± 0.03, p=0.019) but not in RRMS (0.3 ± 0.05) as compared to healthy controls (0.34 ± 0.03, Figure 2.1b) Medication did not have an impact on DC numbers (data not shown).

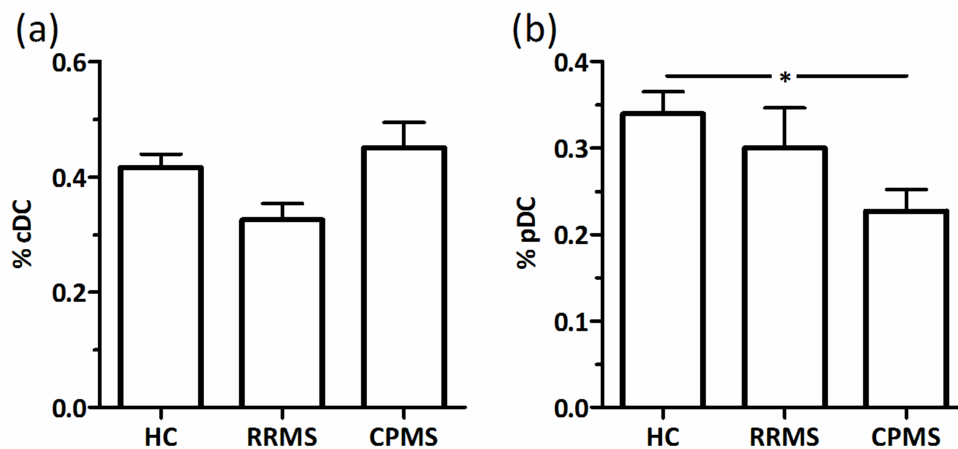

Figure 2.1. Circulating pDC are decreased in CPMS patients (a) Percentage of cDC (BDCA-1$^+$CD14$^-$CD19$^-$) in the mononuclear fraction. (b) Percentage of pDC (BDCA-2$^+$CD14$^-$CD19$^-$) in the mononuclear fraction. Results are shown as mean percentage ± SEM. * P<0.05.

2.3.2 HLA-DRB1*1501 and polymorphisms in the promoter region of IL-7Rα affect the frequency of circulating DC

To investigate whether MS-associated genetic risk factors influence the DC compartment, MS patients and healthy controls were stratified based on the expression of HLA-DRB1*1501. In MS patients, HLA-DRB1*1501 carriers (n=11) had a significantly lower level of circulating cDC (0.27 ± 0.05, p=0.045), but not

of pDC, as compared to non-HLA-DRB1*1501 carriers (n=16, 0.43 ± 0.05) (Figure 2.2). No differences in cDC and pDC percentages were found in HLA-DRB1*1501^{+} versus HLA-DRB1*1501^{-} healthy controls. A significant correlation was found between the lower number of cDC of MS patients and HLA-DRB1*1501 haplotype (p=0.034), whereas this correlation could not be demonstrated in healthy controls. Of interest, a significantly reduced percentage of cDC and pDC was found between MS patients (cDC: p=0.022; pDC: p=0.005) and healthy controls carrying HLA-DRB1*1501.

In addition, MS patients and controls were haplotyped for IL-7Rα. The protective haplotype 2 (hap2) of IL-7Rα is tagged by a T base in exon 6 of the MS-associated SNP (rs6897932). Study subjects were classified according to the presence or absence of hap2. Although no significant differences in cDC or pDC percentages were detected in healthy controls carrying the hap2 compared to non-hap2 carriers, non-hap2 carriers in comparison to hap2 carriers in the MS population have a significantly reduced level of circulating pDC (non-hap2 carriers: n=16, 0.19 ± 0.03, p=0.008), but not cDC, in the peripheral blood (non-hap2 carriers: n=11, 0.33 ± 0.05, Figure 2.3). Whereas a significant association between the lower number of pDC in MS patients and hap2 (p=0.005) was found, this association could not be demonstrated in healthy controls. Moreover, a significantly reduced number of both cDC and pDC was found in non-hap2 carriers when comparing MS patients (cDC: p=0.0038; pDC: p=0.0002) and healthy controls.

These observations show for the first time that certain genetic MS risk factors are associated with differences in the DC compartment.

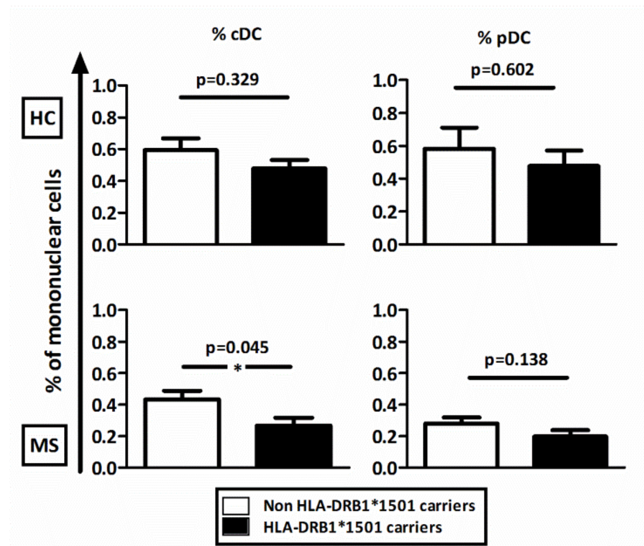

Figure 2.2. blood circulating cDC are decreased in HLA-DRB1*1501^{+} MS patients. The percentage of DC is plotted against the presence or absence of HLA-DRB1*1501 with healthy controls (HC) in the upper panels and MS patients in the lower panels. Results are shown as mean percentage ± SEM. * P<0.05.

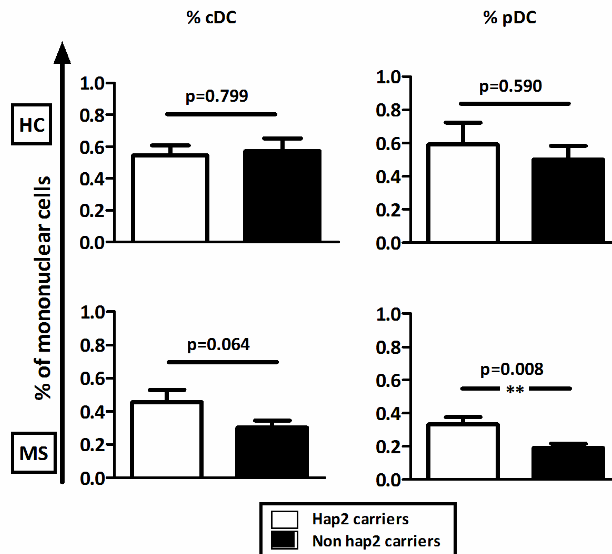

Figure 2.3: Circulating pDC in the blood are decreased in MS patients not carrying haplotype 2. The percentage of DC is plotted against the presence or absence of IL-7Rα haplotype 2 (Hap 2) with healthy controls (HC) in the upper panels and MS patients in the lower panels. Results are shown as mean percentage ± SEM. ** P<0.01.

2.3.3 Circulating DC of MS patients have a migratory and costimulatory phenotype indicative of an inflammatory disease state

In addition to the number of circulating DC, more insights into the role of the different DC subsets in MS can be gained by evaluating their phenotypic profile. To assess the activation state of both DC subtypes, expression of the costimulatory molecules CD80 and CD86 was analyzed by flow cytometry. Both costimulatory molecules on DC provide an important secondary signal to T cells which is needed for their full activation. Our analyses revealed a significantly higher percentage of CD86^{+} cDC circulating in the blood of MS patients (Figure 2.4a), which was found both in the RRMS group (n=29, 0.53% ± 0.14, p=0.04) as in the CPMS group (n=41, 0.71% ± 0.23, p=0.037) as compared to healthy controls (n=65, 0.19% ± 0.05, Figure 2.4a). No significant differences in CD80 expression were detected on cDC of the different study populations (Figure 2.4b), neither did pDC of MS patients show a significant alteration in the expression of CD80 nor CD86 (Figure 2.4c and d). No differences were detected in treated versus untreated MS patients (data not shown).

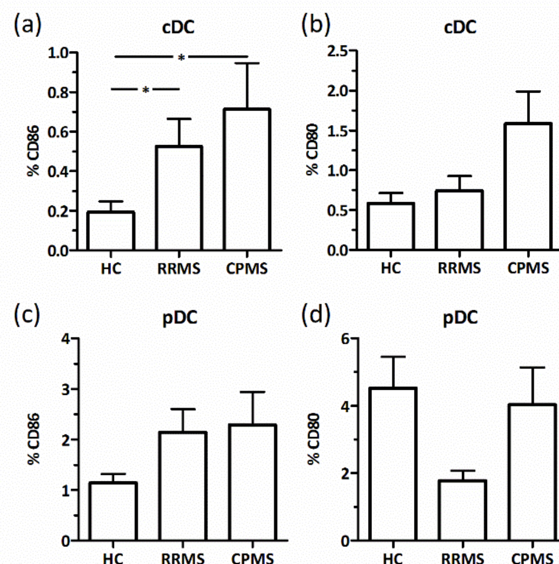

Figure 2.4. Expression levels of CD86 are elevated in cDC of MS patients. The upper panel shows the percentage of BDCA-1^{+} cDC expressing CD86 (a) and CD80 (b). In the lower panel the percentage of BDCA-2^{+} pDC expressing CD86 (c) and CD80 (d) is given. Results are shown as mean percentage ± SEM. * P<0.05.

Next, expression of CD62L, CCR5 and CCR7 was measured on cDC and pDC of MS patients and healthy controls to determine whether circulating DC have an altered migratory profile. Whereas CD62L and CCR7 are important to home to secondary lymphoid tissues, CCR5 is needed to migrate to zones of inflammation. More specifically, in MS it is proven that the CCR5 ligands RANTES and MIP-1β are elevated in the CNS (242, 243). No significant differences in CD62L expression were found on cDC and pDC between MS patients and healthy controls (Figure 2.5a and d). Interestingly, MS patients on IFN-β treatment (n=34) showed a significantly lower number of $CD62L^+$ cDC (69.99% ± 3.13, p=0.038) compared to non-treated MS patients (n=35, 76.47% ± 1.81). A lower precentage of $CD62L^+$ pDC (n=53, 59.85% ± 3.29, p=0.009) was also found in treated versus untreated MS patients (n=35, 69.58% ± 3.57). In addition, a higher number of $CCR5^+$ cDC (n=20, 27.68% ± 3.86, p=0.038) and $CCR5^+$ pDC (n=28, 16.01% ± 2.91, p=0.007) was detected in respectively RRMS and CPMS patients compared to healthy controls (n=48, cDC: 17.58% ± 2.19; pDC: 8.82% ± 1.55, Figure 2.5b and e). RRMS patients showed an upregulation of CCR7 expression on pDC (n=20, 3.29% ± 0.57, p=0.005) as compared to healthy controls (n=49, 1.44% ± 0.3, Figure2. 5f), whereas no alterations in CCR7 expression were detected on cDC (Figure 2.5c). No effect of medication was found for CCR5 or CCR7 expression by DC subsets of MS patients (data not shown).

Since the observed aberrant expression of migratory molecules may account for an enhanced migratory potential of DC in MS, an *in vitro* migration assay was performed to evaluate chemotaxis of purified DC towards a chemokine gradient specific for CCR5 *(i.e.* MIP-1β + RANTES) and CCR7 (*i.e.* CCL19 + CCL21). Migrating cDC towards a CCR7 specific chemokine gradient were observed in 2 out of 3 MS patients (Figure 2.6b). Similar results were observed regarding the migratory potential of pDC from MS patients towards a CCR5 and CCR7 specific chemokine gradient (Figure 2.6c and d).

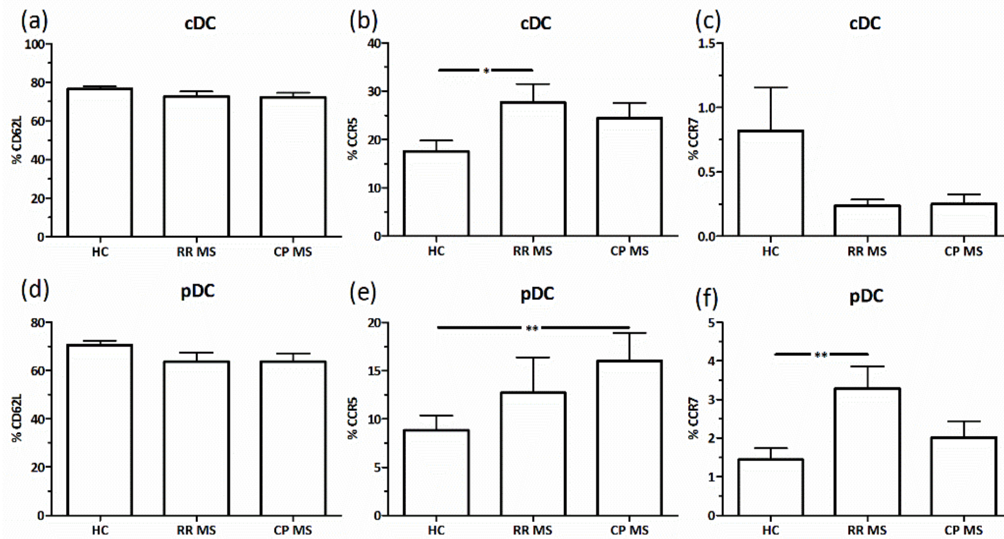

Figure 2.5. Expression levels of migratory markers on DC subsets in peripheral blood are increased during MS. The upper panels show the percentage of BDCA-1^{+} cDC expressing CD62L (a), CCR5 (b) and CCR7 (c). In the lower panel the percentage of BDCA-2^{+} pDC expressing CD62L (d), CCR5 (e) and CCR7 (f) are displayed. Results are shown as mean percentage ± SEM. * $P<0.05$, ** $P<0.01$.

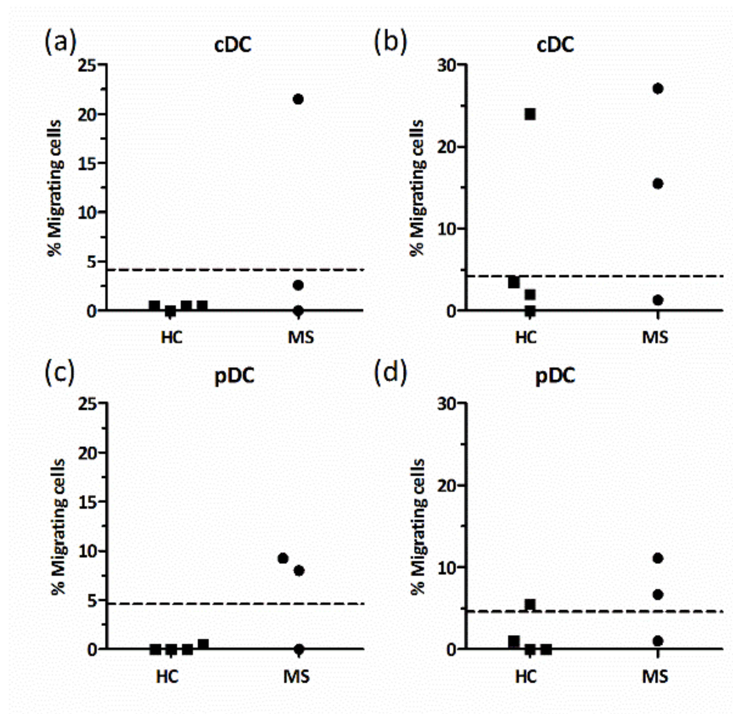

Figure 2.6. cDC and pDC of MS patients show an increased migratory potential towards chemokines specific for CCR5 and CCR7. The upper panel shows the percentage of BDCA-1⁺ cDC migrating towards the CCR5 ligands MIP-1β and RANTES (a) and the CCR7 ligands CCL19 and CCL21 (b). In the lower panels the percentage of migrating BDCA-2⁺ pDC towards MIP-1β and RANTES (c) and CCL19 and CCL21 (d) is shown. A cut off value of 4.2% and 4.6% migrating cells was applied for respectively migrating cDC and pDC (dashed lines) by calculating the mean percentage of migrating cells in the negative control summed with three times the SEM. Results are shown as mean of duplicate measurements for each study subject.

2.3.4 DC of MS patients behave differently after TLR stimulation

To assess the responsiveness of circulating DC to danger signals, blood samples were stimulated overnight with a TLR4 ligand, LPS, combined with IFN-γ or a TLR7 ligand, IQ, in order to induce the secretion of inflammatory mediators by cDC and pDC, respectively (table 2.3). LPS/IFN-γ stimulation significantly induced IL-12p70 production in all subjects. The capacity to induce IL-12p70 secretion was significantly higher in RRMS patients (n=19, 24.8 pg/mL ± 10.25, p=0.024) as compared to age- and gender-matched healthy controls (n=27, 6.65 pg/mL ±

1.32). While no difference in IQ-induced IFN-α secretion was observed between MS patients and healthy controls, a significantly lower baseline level of IFN-α production was apparent in both RRMS and CPMS patients compared to healthy controls (data not shown). Intracellular cytokine staining confirms that circulating DC populations are the main producers of the cytokines detected (Figure 2.7).
Taken together our observations indicate that both cDC and pDC circulating in the peripheral blood of MS patients have a proinflammatory profile and are more prone to migrate towards the site of inflammation.

Table 2.3. IL-12p70 and IFN-α secretion after TLR ligation.

	IL-12p70[a]		**IFNα**[a]	
Group	Mean fold change ± SEM[b]	*P* values[c]	Mean fold change ± SEM[b]	*P* values[c]
HC	6.646±1.32		3.179±1.04	
RRMS	24.795±10.25	**0.024**	5.617±0.36	0.573
CPMS	24.354±9.43	0.203	5.534±1.43	0.583

[a] Blood samples were stimulated overnight with LPS and IFN-γ to test IL-12p70 secretion and IQ stimulation to test IFN-α secretion.
[b] Mean fold changes (±standard error of means) are calculated as results in stimulated condition divided by results in non-stimulated condition.
[c] P values versus healthy controls are shown.

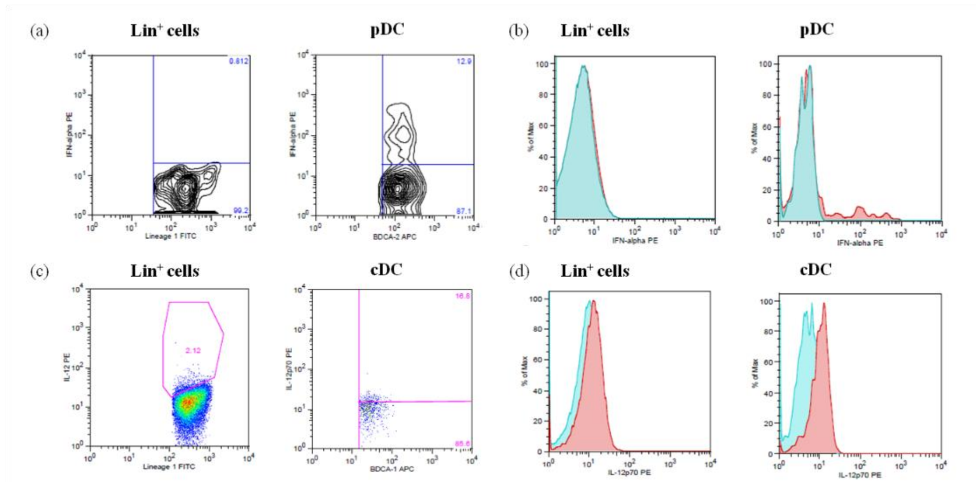

Figure 2.7. **Representative example of intracellular cytokine staining of TLR-stimulated cells.** (a, c) Dot plots are gated on lineage+BDCA- cells (2 panels on the left) and lineage-BDCA+ cells (2 panels on the right) and show IFN-α (a) and IL-12p70 (c) staining (y-axis) versus Lineage/BDCA staining (x-axis). Percentages indicated are within the gated population. (b) Histogram overlays show IFN-α stained IQ-stimulated lin-BDCA-2+ cells (red histogram) compared to non-stimulated cells (green histogram). (d) Histogram overlays show IL-12p70 stained IFNγ/LPS-stimulated lin-BDCA-2+ cells (red histogram) compared to non-stimulated cells (green histogram).

2.4 Discussion

Given the important role of DC in polarizing and controlling the T cell response, we hypothesized that a differential distribution and aberrant function of DC in MS affect homeostasis between immunity and tolerance and influence the susceptibility to disease and/or its course. A higher expression of the costimulatory molecule CD86 as well as the significantly higher induction of IL-12p70 following TLR ligation indicate that cDC of RRMS patients are in a more activated, *i.e.* immunogenic, state as compared to healthy controls, thereby confirming previous studies by others (176, 177, 244). No differences regarding the expression of CD80 and CD86 by pDC or secretion of IFN-α upon TLR challenge could be demonstrated between MS patients and healthy controls, although MS-derived pDC secrete significantly lower baseline levels of IFN-α as compared to healthy controls, which is in line with previous reports (244). Secretion of IFN-α by pDC shapes the immune response, thereby influencing other regulatory cells, including cDC. Noteworthy, at least two different subsets of pDC were demonstrated by Schwab *et al. (245)*. Type 1 pDC ($CD123^{hi}$ $CD86^{dim}$ $TLR2^{dim}$) are the main source of IFN-α and induce IL-10-producing T cells. In contrast, type 2 pDC ($CD123^{dim}$ $CD86^{hi}$ $TLR2^{hi}$) secrete IL-6 and TNF-α and direct naive T cells towards IL-17-secreting Th17 cells. Interestingly, the authors demonstrated a reduced ratio of Type 1/Type 2 pDC in MS patients. In agreement with these findings, we report significantly reduced levels of IFN-α in MS patients, albeit we did not investigate the relative contribution of both pDC subsets. Hence, the impaired function of pDC in MS may interfere with the normal immunoregulatory mechanisms.

Our results revealed a significantly higher expression of the migration-associated marker CCR5 on cDC of RRMS patients. Since others have reported upregulated expression of CCR5 ligands RANTES and MIP-1β in MS lesions (242, 243), this may suggest an increased influx of cDC into the CNS. Indeed, Pashenkov et al. (177) found a correlation between CCR5 levels on cDC and their numbers in the cerebrospinal fluid (CSF), which together with our data demonstrating DC migrating towards a CCR5 specific chemokine gradient, confirms that cDC of MS patients show migration capability to the CNS. No differences regarding the expression of CD62L and CCR7 on cDC could be detected between MS patients and healthy controls indicating that cDC are still able to migrate to the lymph

nodes to activate T cells. In contrast, pDC of MS patients display increased CCR7 expression, suggesting an enhanced migration of pDC to secondary lymphoid organs and more specifically to the T cell zones of the lymph nodes (246). Our results indicate increased migration towards CCL19 and CCL21 by pDC in MS patients. Moreover, the increased expression of CCR5 on pDC in CPMS patients together with the migration capacity towards MIP-1β and RANTES suggest an enhanced migration to the CNS. Although the group of Pashenkov et al. indicated that pDC recruitment in the CNS was CCR5 independent, they did not distinguish between RRMS and CPMS.

While we did not detect differences in the percentage of circulating cDC in MS patients versus controls, we observed a reduced cDC blood cell number in HLA-DRB1*1501^{+} compared to HLA-DRB1*1501^{-} MS patients. HLA-DRB1*1501 has been reported to be the main restriction element for presenting the immunodominant epitope of myelin basic protein (MBP_{84-102}) (71). Since cDC are especially important in antigen presentation, the expression of HLA-DRB1*1501 may thus affect the strength, specificity and duration of DC-T cell interactions. The reduced levels of cDC observed in the blood of DRB1*1501 carriers may reflect enhanced trafficking from the blood towards the CNS, which together with the increased activation state of cDC may drive the inflammatory response in MS. In line with this, Sombekke et al. showed that HLA-DRB1*1501 carriers have more focal abnormalities in the spinal cord (247) and other studies indicated that HLA-DRB1*1501 is associated with a more severe disease course (247, 248). Additional contributing factors are likely to be involved since circulating DC subsets were still significantly lower when comparing HLA-DRB1*1501^{+} MS patients with healthy HLA-DRB1*1501^{+} carriers.

No alterations in pDC numbers were found in RRMS, in line with previous studies (176, 249). We demonstrated a decrease in pDC numbers in CPMS patients. Although reduced percentages of pDC are also reported in other autoimmune diseases, such as systemic lupus erythematodes and rheumatoid arthritis (250, 251), previous studies only investigated pDC numbers in RRMS (178, 249). We show for the first time that pDC of MS patients not carrying the protective haplotype 2 IL7Rα allele were decreased in the blood compared to carriers. This may suggest enhanced migration towards lymph nodes and the CNS.

Finally, and to our knowledge for the first time, our data suggest that treatment of MS is associated with changes in the DC compartment: a lower number of

CD62L$^+$ cDC and pDC was found in treated patients. This suggests a mechanism for decreased migration of DC, which would reduce their proinflammatory state. It also suggests that treatment of MS may not only affect T lymphocytes but also DC and bring about at least part of its activity through DC.

3

Tolerogenic dendritic cells generated by in vitro treatment with SAHA are not stable in vivo

Based on:

Tolerogenic dendritic cells generated by in vitro treatment with SAHA are not stable in vivo.

Thewissen K[1], Hendriks JJA[1], Vanhees M[2], Stinissen P[1], Slaets H[1], Hellings N[1]

Submitted

[1] Biomedical Research Institute, Hasselt University, Belgium
[2] Department of Clinical Chemistry, Microbiology and Immunology, Ghent

ABSTRACT

The aim of this chapter is to examine whether the histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA) can generate stable tolerogenic dendritic cells (DC) to counteract autoimmune responses in an animal model of multiple sclerosis. We investigated if the potency of tolerogenic DC could be increased by continuous treatment during the differentiation towards DC compared to the standard 24h treatment of already terminally differentiated DC. SAHA (vorinostat) was already shown to induce a less severe EAE course when directly administered *in vivo* SAHA was able to suppress costimulatory molecules on DC leading *in vivo* to reduced Th1 and Th17 polarizing cytokines (225). In line with the important role of SAHA on DC in the described effect, we first investigate if the potency of these tolerogenic DC can be increased by continuous treatment during *in vitro* differentiation towards DC in comparison to the standard 24h treatment of already differentiated DC. We show that *in vitro* treatment with SAHA reduces the generation of new CD11c^{+} DC out of mouse bone marrow. Newly generated DC under SAHA treatment possess increased characteristics of tolerogenic DC. More specifically, they show reduced antigen-presenting function as evidenced by a reduction in myelin endocytosis, a decreased MHC-II expression and a failure to upregulate costimulatory molecules upon LPS challenge. SAHA-generated DC display a reduction in proinflammatory cytokines and molecules involved in apoptosis-induction, inflammatory migration and TLR signaling. We demonstrated that the underlying mechanism of tolerance induction by SAHA involves a diminished STAT1 phosphorylation and was independent of STAT6 activation. Although *in vitro* results were promising, SAHA-generated tolerogenic DC were not able to alleviate the development of experimental autoimmune encephalomyelitis in mice. *In vitro* wash out experiments demonstrated that the tolerogenic phenotype of SAHA-treated DC is reversible.
Taken together, while SAHA potently boosts tolerogenic properties in DC during the differentiation process *in vitro*, SAHA-generated DC were unable to reduce autoimmunity *in vivo*. Our results imply that caution needs to be taken when developing DC-based therapies to induce tolerance in the context of autoimmune disease.

3.1 Introduction

Histone deacetylases (HDAC) and their counterparts, histone acetyl transferases (HAT) determine the acetylation status of histone proteins (252, 253). They also target non-histone proteins, such as transcription factors, chaperone proteins, signal transduction mediators and DNA repair enzymes (254). Acetylation of these proteins influences protein function, signaling, stability and protein/protein-DNA interactions (255). In this way, cellular biology is heavily affected by the use of HDAC inhibitors. By regulating the natural process of acetylation and deacetylation, HDAC inhibitors selectively alter gene expression. This not only makes them promising anticancer drugs, recent evidence indicates that HDAC inhibitors could be of use in autoimmune and inflammatory diseases (256-259). In these studies, the observed therapeutic effect was ascribed to a reduction in circulating inflammatory cytokines and possible immunomodulatory function of these drugs. The exact molecular and cellular mechanisms however remain elusive. One of the immune cell types affected by the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) are dendritic cells (DC). Reddy et al. demonstrated that the expression of costimulatory molecules CD40 and CD80 was reduced on DC when treated for 16-18h with the HDAC inhibitor SAHA. Other studies revealed that 24h SAHA treatment affects DC functionality through inhibition of DC-directed Th1 and Th17 cell-polarization and suppression of cytokines (257, 259, 260). We hypothesize that the potency of SAHA to generate tolerogenic DC can be boosted when DC are continuously treated during their differentiation out of bone marrow cells (10d treatment) and that these SAHA-generated DC have therapeutic potential in autoimmune disease.

Since DC have the ability to shape the immune response by polarizing T cells into different effector cell types, there is significant interest to use DC to limit immunopathogenic responses during transplantation, allergy and autoimmune disease. In regard to this, SAHA decreased *in vivo* alloproliferation in an acute graft-versus-host-disease mouse model (258). Injection of antigen-pulsed tolerogenic DC may thus deliver immunoregulatory peptides *in vivo* leading to the induction of antigen specific regulatory T cells or inhibition of autoreactive T cells (261, 262). To date however, there is no consensus on which stimuli guarantee the induction of stable tolerogenic DC. Unstable tolerogenic DC could reverse back *in vivo* to an immune stimulatory phenotype when in contact with proinflammatory

stimuli and may thus provoke undesirable responses. It is therefore critically important to thoroughly test the potency and durability of tolerance inducing drugs.

The goal of this study was to augment the immunomodulatory and tolerogenic capability of the HDAC inhibitor SAHA to generate stable tolerogenic CD11c^{+} DC that counteract autoimmune responses in an animal model of multiple sclerosis (MS). SAHA-mediated effects on DC phenotype and function and the mechanisms involved were assessed *in vitro*. Consequently, the disease modifying potential of SAHA-generated CD11c^{+} DC was tested in mice with experimental autoimmune encephalomyelitis (EAE).

3.2 Materials and methods

3.2.1 Mice

Eight week old C57Bl/6J female mice were purchased from Harlan (Horst, the Netherlands). Animals were housed under standard conditions with water and food ad libitum in the animal facility of the Biomedical Research Institute of Hasselt University. The animals were allowed to acclimatize one week before the start of the experiments. Experiments were conducted in accordance with institutional guidelines and approved by the local Ethical Committee for Animal Experiments of Hasselt University.

3.2.2 DC generation, culture and treatment

DC were generated out of bone marrow isolated from tibiae and femurs of C57Bl/6J female mice based on Inaba et al. and Lutz et al. (263, 264). Briefly, bone marrow was rinsed with IMDM (Lonza, Braine-L'Alleud, Belgium) supplemented with 10% FCS (GIBCO, Gent, Belgium). After centrifugation the pellet was resuspended in red blood cell lysis buffer and incubated for 10 min at room temperature. Next, cells were placed in IMDM supplemented with 10% FCS, 20 ng/ml GM-CSF (R&D, Abingdon, UK), 10 ng/ml IL-4 (R&D) and 1% penicillin-streptomycin (GIBCO) and consequently plated at $3x10^6$ cells/well and incubated at 37°C and 5% CO_2. Culture medium was changed at day 3, 6 and 8. On day 6, cells were harvested with 5mM EDTA (VWR, Leuven, Belgium), counted and replated at 500 000 cells/well. DC or bone marrow were treated for 24h (standard treatment) or 10d (from start of differentiation process) with 0.5 or 1 µM SAHA (Cayman Chemical Company, Huissen, the Netherlands). To generate mature DC, cells were stimulated 24h with 100 ng/ml LPS (Sigma Aldrich, Bornem, Belgium). To assess the durability of the SAHA effect, DC were treated for 10d with SAHA, consequently placed on culture medium without SAHA and finally restimulated with LPS on day 12 of culture. SAHA was dissolved according to manufacturer's instruction in DMSO, aliquoted and stored at -20°C. Prior to use SAHA was diluted to the appropriate concentration in IMDM. Sterile DMSO was used as the diluent control. Morphology and purity of cell cultures are displayed in figure 3.1.

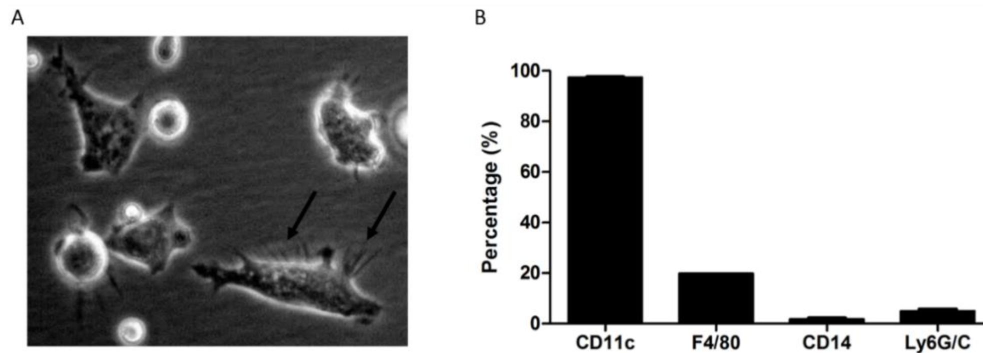

Figure 3.1. Morphology and purity of cell cultures. A) Morphology of cultured CD11c$^+$ DC observed with Zeiss phase contrast microscopy (optics 40x). Arrows indicate typical dendrites on DC. B) Purity of cell cultures determined with flow cytometry. DC, macrophages, monocytes and granulocytes were measured with antibodies against respectively CD11c, F4/80, CD14 and Ly6G/C. F4/80, CD14 and Ly6G/C were measured within the CD11c$^+$ population.

3.2.3 Cell surface phenotype analysis

DC were harvested and stained with CD80-FITC, CD86-PE, PD-L1-PE, FasL-PE, CCR5-PE, TLR4-PE, TLR-9 or I-Ab-PE (eBioscience, Vienna, Austria) combined with CD11c-APC (BD Biosciences, Erembodegem, Belgium). Data was acquired using the FACS Aria II (BD Biosciences) and analyzed with the FacsDiva software (BD Biosciences). As gating strategy, a gate was set on the CD11c$^+$ population. Expression levels of surface molecules were analyzed within this DC gate.

3.2.4 Myelin phagocytosis

Endocytosis was determined by measuring the cellular uptake of 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI, Sigma Aldrich) labeled myelin. DC were incubated for 90 minutes with 20 µg/ml DiI-myelin at 37°C and 5% CO_2. Non-ingested myelin was removed by washing the plates with PBS (Lonza). To determine endocytosis specifically within the DC population, a CD11c-APC staining was performed. Cells were analyzed by FACS Aria II (BD Biosciences) as described above. The mean fluorescence intensity (MFI) in the PE channel was used as a measure for the uptake of labeled myelin.

3.2.5 Cytokine ELISA

Secretion of IL-6, IL-10 and TNF-α was measured in the supernatant of DC cultures using ELISA, following manufacturer's protocol (eBioscience). Plates were read at a wavelength of 450 nm (reference filter 630 nm) using a Bio-Rad Benchmark spectrophotometer (Bio-Rad Laboratories, Eke, Belgium).

3.2.6 STAT signaling

After 10 days of culture, DC were allowed to rest on IMDM for 2 hours at 37°C and 5% CO_2. Cells were incubated for 15 minutes with IMDM supplemented with 10% FCS, 20 ng/ml GM-CSF, 10 ng/ml IL-4 and 1% penicillin-streptomycin. Mature DC also in addition received 100 ng/ml LPS. After the 15 min, cells were immediately fixed by adding cytofix buffer (BD Biosciences) for 10 minutes at 37°C. Cells were stained with CD11c-APC and then permeabilized by adding Perm Buffer III (BD Biosciences) for 30 minutes on ice. Phosphorylation of STAT1 (pY701) and STAT6 (pY641) was determined by staining with respectively PE-pSTAT1 or PE-pSTAT6 antibodies (both obtained from BD Biosciences) for 60 minutes at room temperature. Data was acquired using the FACS Aria II and analyzed with the FacsDiva software. Phosphorylation of STAT1 and STAT6 was analyzed within the $CD11c^+$ DC gate.

3.2.7 EAE induction and treatment with DC

EAE was induced in ten week old C57Bl/6J female mice from Harlan using the Hooke Kit™ (Hooke Laboratories, Lawrence, USA) according to manufacturer's instructions. Briefly, mice were injected subcutaneously with 100 µl of 1 mg/ml myelin oligodendrocyte protein peptide $(MOG)_{35-55}$ emulsified in CFA containing 2 mg/ml mycobacterium tuberculosis H37Ra. Mice received an intraperitoneal injection of 100 µl of 2 µg/ml pertussis toxin directly after immunization and 24h later. DC were treated with 1 µM SAHA for 10d. Next, DC were pulsed for 24h with 5 µg/ml MOG_{35-55} to generate MOG-presenting DC. Mice were then treated with intraperitoneal injections of $1x10^6$ MOG-pulsed DC, SAHA-treated MOG-pulsed DC or PBS (control) at following time points: 3 days before EAE induction and 3, 6 and 9 days post induction. Mice were evaluated on a daily basis for changes in body weight and disease severity, using a standard 5-point scale: 0, no clinical symptoms; 1, decreased tail tone; 2, paresis of hind limbs; 3, paralysis of hind limbs; 4, quadraparesis; 5, death.

3.2.8 MOG specific immune cell proliferation assay

At day 9 or 11 post induction, spleens and inguinal lymph nodes were isolated from EAE mice. Single-cell suspensions were obtained by passage through a 70 µM-mesh (BD). Spleen cells underwent a Ficoll density gradient centrifugation after which the buffy layer was collected and washed with PBS. Lymph node and spleen cells were placed in RMPI-1640 (Lonza) medium containing 2% mouse serum (Harlan), 20 µM 2-mercaptoethanol (Sigma Aldrich), 1% non-essential amino acids (Sigma Aldrich), 1% sodium pyruvate (Sigma Aldrich) and 1% penicillin-streptomycin and restimulated with 20 µg/ml MOG. Unstimulated cells were used as a control. 5 µCi (^{3}H)-thymidine (Perkin-Elmer, Zaventem, Belgium) was added after 2 days of culture and cells were harvested 24h later using an automatic cell harvester (Pharmacia, Uppsala, Sweden). Radioactivity was quantified by means of a β-plate liquid scintillation counter (Perkin-Elmer). Stimulation indexes were calculated by dividing proliferation values in the MOG stimulated cultures by those in the respective non-stimulated controls.

3.2.9 Statistics

Data were statistically analyzed using GraphPad (Software Prism, La Jolla, CA, USA) and are presented as mean ± SEM. Normality was checked with a D'Agostino & Pearson omnibus normality test. One-way ANOVA followed by a Tukey post-test for testing individual groups were performed. For EAE experiments a two-way ANOVA was used dependent on time and treatment followed by a Bonferroni test to examine significance between treatment groups and the control PBS group. Significance was considered positive when p value were <0.05.

3.3 Results

3.3.1 SAHA treatment delays the generation of bone marrow derived CD11c$^+$ DC

We first investigated the effect of SAHA on the *ex vivo* generation of DC from mouse bone marrow precursors by measuring CD11c expression in 10 day cultures. We tested the effect of SAHA either directly on already differentiated CD11c$^+$ DC (standard treatment) or on the differentiation towards DC (treatment during the complete culture period, 10d). A low dose (0.5 or 1µM) of SAHA was chosen since higher concentrations (2.5-7.5µM) of SAHA arrest cell growth (265). No increase of apoptosis was detected based on cell morphology and cell counts in tested conditions (data not shown). To generate mature CD11c$^+$ DC, an LPS stimulus was applied during the last 24h of culture. When measuring the CD11c expression in standard SAHA-treated DC a modest but significant reduction of CD11c was observed in the 1µM SAHA treated group compared to the corresponding control group (Fig. 3.2A). In contrast, a strong reduction in the percentage of CD11c$^+$ DC was demonstrated when bone marrow was treated with 0.5 or 1µM SAHA during the whole differentiation process towards CD11c$^+$ DC (10d, Fig. 3.2B). These results indicate that standard SAHA treatment only slightly affects already differentiated DC, whereas continuous treatment can significantly limit the generation of new CD11c$^+$ DC out of bone marrow precursors.

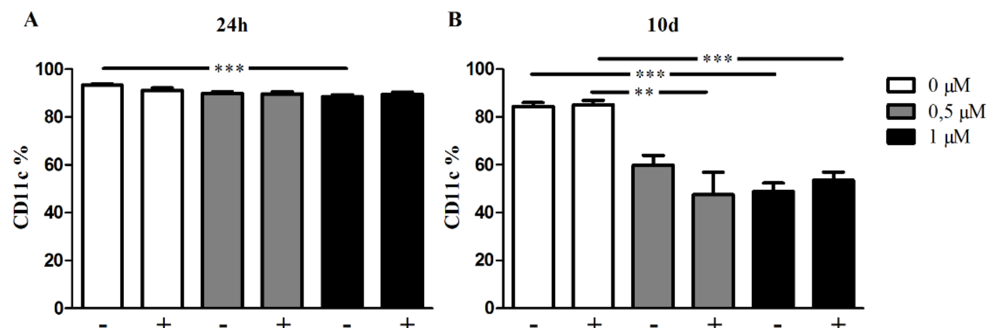

Figure 3.2. 10 days SAHA treatment limits the differentiation of CD11c⁺ DC. Primary immature DC were cultured out of mouse bone marrow in IMDM with GM-CSF and IL-4 for 10 days. Mature DC were generated by adding LPS during the last 24 hours of culture. Flow cytometry of CD11c expression on DC treated with SAHA for (A) 24h or (B) 10d. Results are shown as mean percentage ± SEM. n = 13-22, - or + is with or without LPS, ** $p<0.01$ & *** $p<0.001$

3.3.2 SAHA-treated CD11c⁺ DC fail to upregulate costimulatory molecules after LPS stimulation

We next tested whether SAHA-treated CD11c⁺ DC are able to provide costimulation when maturation is induced by LPS. Costimulation is one of the important signals a DC delivers to generate effective T-cell responses.

Untreated DC significantly upregulated both CD80 and CD86 after 24h of LPS stimulation ($p<0.001$, Fig. 3). This upregulation could not be blocked by the standard SAHA treatment (Fig. 3.3A&B). In contrast, upregulation of CD80 expression was blocked by SAHA treatment for 10d (Fig. 3.3C). In addition, a significant inhibition of LPS-induced CD86 upregulation was detected when CD11c⁺ DC were treated with 1μM SAHA (Fig. 3.3D). The inhibition of the costimulatory molecules CD80 and CD86 in SAHA-generated DC is a first indication that continuous SAHA treatment during differentiation of CD11c⁺ DC is much more efficient in boosting tolerogenic characteristics, compared to the standard treatment of already differentiated DC.

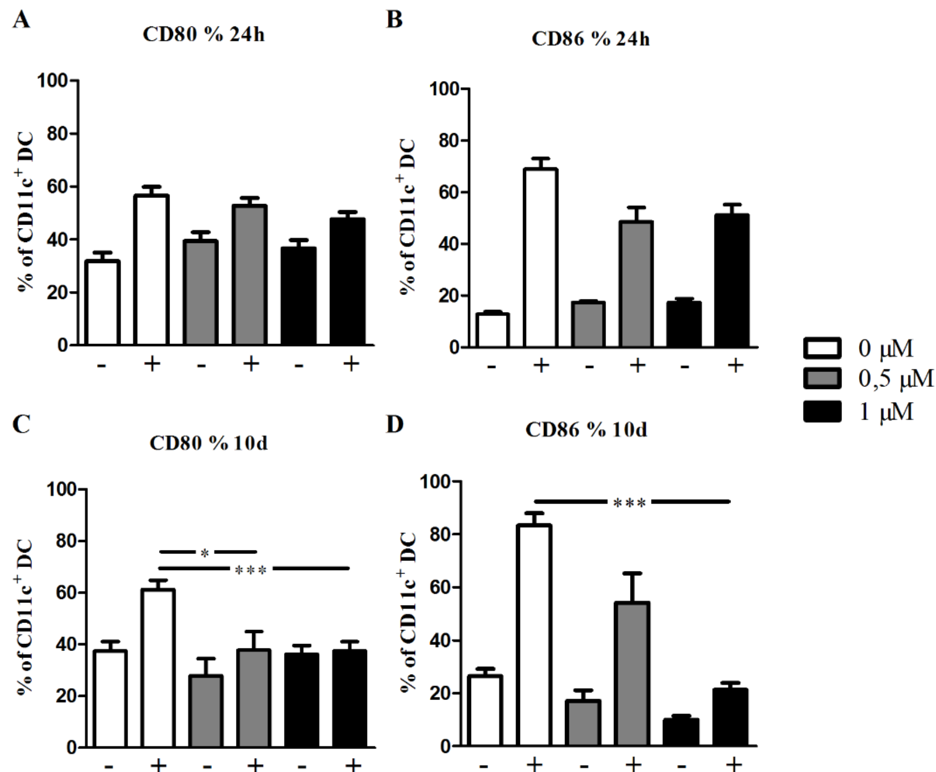

Figure 3.3. SAHA treatment of 10 days blocks the costimulatory upregulation of CD80 and CD86 after LPS stimulation. Primary immature DC were cultured out of mouse bone marrow in IMDM with GM-CSF and IL-4 for 10 days. Mature DC were generated by adding LPS for 24 hours. Flow cytometry of CD80 (A) and CD86 (B) on 24h SAHA-treated DC. Flow cytometry of CD80 (C) and CD86 (D) on 10d SAHA-generated DC. Results are shown as mean percentage ± SEM. n = 13-18, - or + is with or without LPS, * $p<0.05$ & *** $p<0.001$.

3.3.3 SAHA-generated CD11c^{+} DC downregulate the expression of molecules involved in apoptosis-induction, migration and TLR signaling

Next, we investigated whether SAHA-generated DC undergo other phenotypical changes. Here to, we analyzed molecules involved in apoptosis-induction (PD-L1, FasL), migration (CCR5) or TLR signaling (TLR4, TLR9). While SAHA (1 µM) significantly reduced the expression of FasL (Fig. 3.4A), PD-L1 was not affected by SAHA (Fig. 3.4B), indicating that the HDAC inhibitor influences specific cell

death inducing pathways. Moreover, migratory properties of SAHA-generated DC may be influenced, as was detected by a significant diminished CCR5 expression after SAHA treatment (Fig. 3.4C). In addition, we detected a reduced cell surface expression of TLR4 and TLR9 (Fig. 3.4D&E) making SAHA-generated CD11c$^+$ less prone to activation by danger signals. The reduction in TLR expression combined with the block in upregulation of costimulatory molecules (see above) suggests generation of stable tolerance-inducing DC unresponsive to factors that induce an immunogenic shift in DC function when SAHA was added during the entire DC differentiation process.

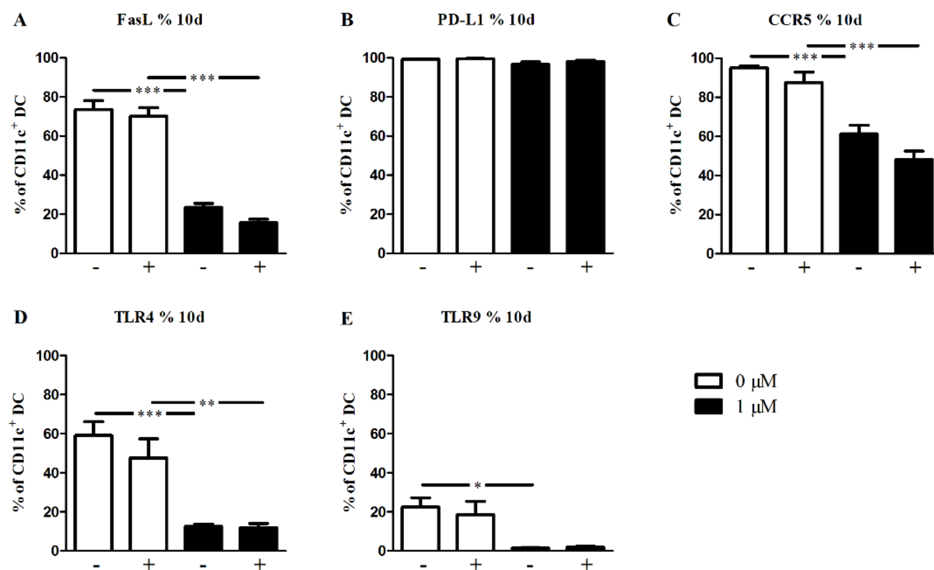

Figure 3.4. SAHA treatment of 10 days reduces the expression of FasL, CCR5, TLR4 and TLR9 on CD11c$^+$ DC. Primary immature DC were cultured out of mouse bone marrow in IMDM with GM-CSF and IL-4 for 10 days in the presence or absence of SAHA followed by flow cytometric analysis of the respective proteins. Mature DC were generated by adding LPS for 24 hours. Flow cytometry of FasL (A), PD-L1 (B), CCR5 (C), TLR4 (D) and TLR9 (E) on 10d SAHA-generated DC. Results are shown as mean percentage ± SEM. n = 4, - or + is with or without LPS, * $p<0.05$, ** $p<0.01$ & *** $p<0.001$.

3.3.4 SAHA-treated CD11c$^+$ DC produce less pro-inflammatory cytokines after LPS stimulation

The cytokines produced by DC play a pivotal role in the T-cell polarization. Therefore supernatants of standard or 10d SAHA-treated DC cultures were

analyzed with ELISA for several cytokines, namely IL-6, TNF-α and IL-10. Standard treatment of $CD11c^{+}$ for 24h with 1µM SAHA significantly reduced the production of IL-6 and TNF-α after LPS stimulation (Fig. 3.5A-B). In contrast no changes on the level of the anti-inflammatory cytokine IL-10 were detected (Fig. 3.5C). Similar results were obtained when DC were generated in the presence of SAHA. IL-6 secretion was significantly reduced with a dose of 1µM, whereas TNF-α could be decreased with a minimum dose of 0.5µM (Fig 3.5D-E). As was the case with standard SAHA treatment, no change in IL-10 production was observed (Fig 3.5F).

Altogether, SAHA treatment diminished the production of pro-inflammatory cytokines, whereas the anti-inflammatory cytokine IL-10 remained on the same level. Reduced secretion of pro-inflammatory cytokines upon TLR ligation is another hall mark of tolerogenic DC which in our study was previously suggested based on SAHA effect on costimulation and TLR expression.

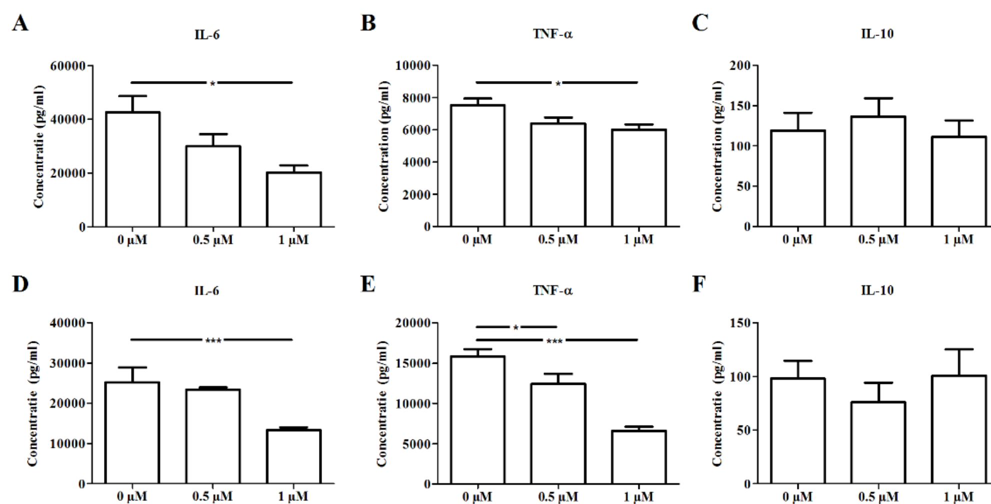

Figure 3.5. DC cell cultures treated with SAHA produce less pro-inflammatory cytokines after LPS stimulation. Production of IL-6 (A), TNF-α (B) and IL-10 (C) in response to LPS was measured with ELISA in the supernatant of cell cultures treated with 24h SAHA . Production of IL-6 (D), TNF-α (E) and IL-10 (F) in response to LPS were measured with ELISA in the supernatant of cell cultures treated for 10d with SAHA. Results are shown as mean percentage ± SEM. n = 9-14, * $p<0.05$ & *** $p<0.001$.

3.3.5 Endocytosis of myelin and MHC-II expression is diminished after 10d SAHA

Providing costimulation and cytokines are important functions, but DC are foremost specialized professional antigen-presenting cells. It is reported that immature DC are exceptional in phagocytozing antigens from the environment, whereas the actual processing and presentation is done by mature DC (173, 175, 266). Based on the observation that 10d SAHA treatment potently inhibited upregulation of costimulatory molecules compared to the standard treatment, we investigated whether 10d SAHA treatment also had an effect on myelin phagocytosis and the expression of MHC class II molecules. Myelin uptake of DC is evident in post-mortem brain of MS patients suggesting DC recruitment and maturation in MS lesions. Therefore, when self-antigens are made available *in vivo* as a result of myelin damage, DC can take up myelin antigens and contribute to the local activation and expansion of pathogenic T cells (267). To investigate myelin uptake, DiI-labeled myelin was added for 90 minutes to the cell cultures before flow cytometric analysis. In addition, the expression of mouse MHC-II (I-Ab) was measured. LPS matured $CD11c^+$ DC showed reduced myelin uptake compared to immature $CD11c^+$ DC (Fig. 3.6A). Interestingly, SAHA significantly reduced the uptake of DiI-labeled myelin regardless of maturation state. Furthermore, mature $CD11c^+$ DC show increased MHC-II levels in comparison with immature $CD11c^+$ DC (Fig. 3.6B). MHC-II expression was almost completely absent after 1µM SAHA treatment.

Thus, antigen-uptake and presentation is downregulated by 10d SAHA treatment. Together with the reduced induction of costimulatory molecules and proinflammatory cytokines, SAHA-generated DC seem less capable to interact with and stimulate T cells.

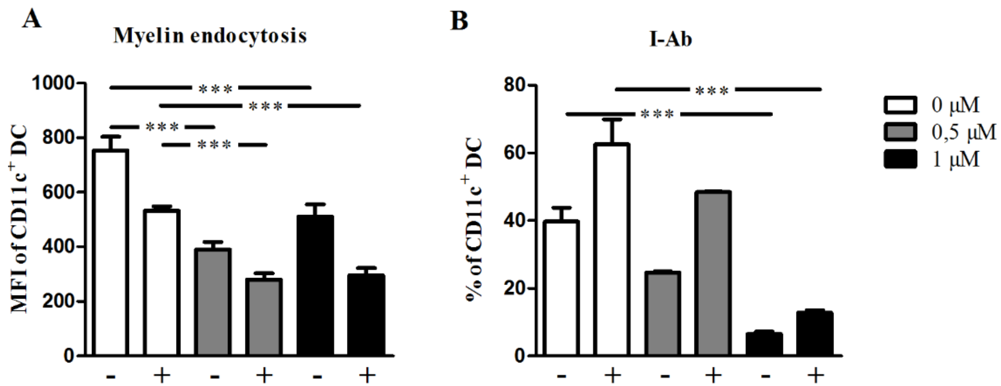

Figure 3.6. CD11c⁺ DC-generated for 10d with SAHA take up less myelin and show reduced MHC-II levels. A, DiI-labeled myelin was added 90 minutes to the cell cultures prior to flow cytometry. B, MHC-II or I-Ab expression was simultaneously measured with flow cytometry. Results are shown as mean MFI or percentage ± SEM within the CD11c+ gate n = 4-6, - or + is with or without LPS, *** p<0.001.

3.3.6 SAHA effects coincides with reduced STAT1 signaling and are independent of STAT6 signaling

To reveal the downstream targets of SAHA, STAT phosphorylation is measured in 10d SAHA-treated and untreated $CD11c^+$ cultures. DC maturation requires activation of the STAT1 pathway, whereas immature DC have constitutive activation of STAT6 signaling (268). SAHA reduced STAT1 phosporylation at Y701 compared to the corresponding control condition in agreement with the above described block in DC maturation (Fig.3.7A). In contrast, STAT6 phosphorylation (pY641) was reduced after LPS stimulation regardless of the presence of SAHA (Fig.3.7B).

These results suggest that SAHA, when added at the start of differentiation, blocks $CD11c^+$ DC maturation at least partially by interfering with STAT1 phosphorylation and consequently activation.

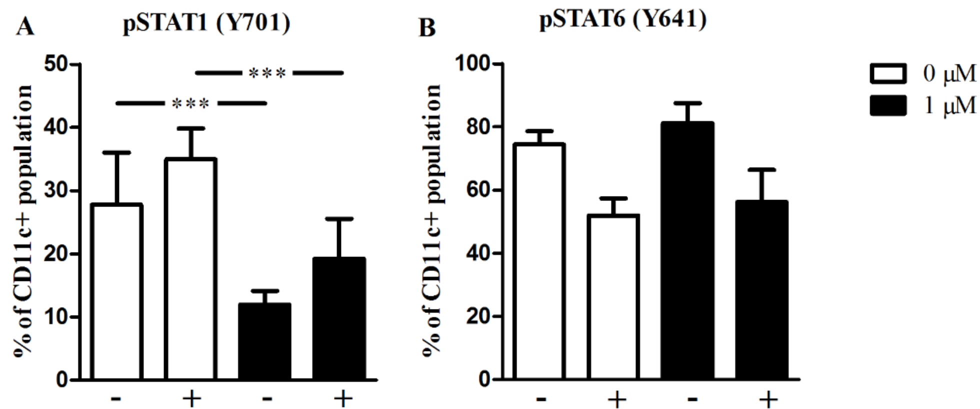

Figure 3.7. SAHA blocks maturation of CD11c⁺ DC through diminished phosphorylation of STAT1 (pY701) and independent of STAT6 phosphorylation (pY641). Results are shown as mean percentage ± SEM. n = 4, - o + is with or without LPS, *** $p<0.01$.

3.3.7 SAHA treatment did not enhance clinical potency of MOG-pulsed $CD11c^+$ DC in EAE

To test whether SAHA-generated $CD11c^+$ DC have disease modulatory effects in vivo, $1x10^6$ MOG-pulsed $CD11c^+$ DC pretreated in vitro with 1μM SAHA were injected into EAE mice.

Injections of mice with MOG-pulsed DC modestly but significantly reduced disease symptoms during 3 consecutive days compared to PBS injected control mice. No differences in disease course were detected between SAHA-generated and untreated MOG pulsed DC (Fig. 3.8A). Moreover from day 16 onward, all groups followed the same clinical course suggesting only a transient effect of the DC if any.

To test whether MOG-specific T-cells responses were affected by DC treatment of EAE mice, lymph nodes and spleen were collected. Mice injected with 10d SAHA-generated or untreated DC equally reduced MOG responses in spleen compared with PBS injected controls (Fig. 3.8C). Untreated MOG-pulsed $CD11c^+$ DC showed also significant less MOG reactivity in lymph nodes (Fig. 3.8B). Together, these analyses explain the slight delay in onset of clinical EAE scores.

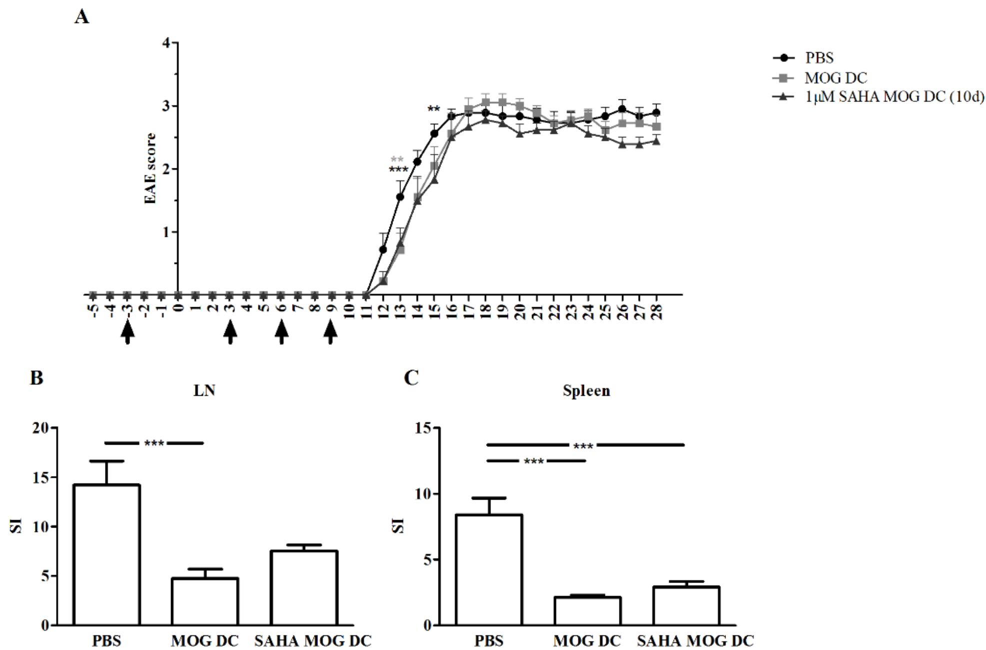

Figure 3.8. MOG-pulsed DC reduced disease symptoms independent of SAHA treatment during 3 consecutive days. A, Mice were injected intraperitoneally on 4 time points; -3d 3d, 6d and 9d post-induction with PBS, $1x10^6$ MOG-pulsed $CD11c^+$ DC treated with 1µM SAHA for 10d (n = 10 animals/group). B, Stimulation index of MOG responses of lymph node cells ((^3H)-thymidine labeled) isolated on day 11 out of mice injected with PBS, $1x10^6$ MOG-pulsed or MOG-pulsed 1µM SAHA-treated $CD11c^+$ DC (10d treatment). C, Stimulation index of MOG responses of spleen cells ((^3H)-thymidine labeled) isolated on day 11 out of mice injected with PBS, $1x10^6$ MOG-pulsed or MOG-pulsed 1µM SAHA-treated $CD11c^+$ DC (10d treatment). Results are shown as mean EAE score or SI ± SEM. n = 7-10, SI = Stimulation Index, LN = Lymph nodes, ** p<0.01, *** p<0.001.

3.3.8 Ex vivo SAHA-generated tolerogenic DC phenotype is not stable

Given the transient effect on the EAE course, the question was raised whether *in vitro* $CD11c^+$ DC treated with 1µM SAHA for 10d retain their tolerogenic characteristics once injected *in vivo*. To address this hypothesis, cell cultures were set up with 10d treatment of SAHA, but before analysis cells were placed in culture medium for 2 days without SAHA. Next maturation was induced by adding LPS for 24h and induction of co-stimulatory molecules were analyzed as described above. This wash out experiment indicated that the DC regained the ability to upregulate the costimulatory molecules CD80 and CD86 in response to LPS (Fig. 3.9). CD86

was still significantly lower than untreated controls. However the previous observed block (Fig. 3.3D) was abolished.

From these experiments, it can be concluded that after removal of SAHA, $CD11c^+$ DC overcome the induced block during their differentiation process and regain their costimulatory potential.

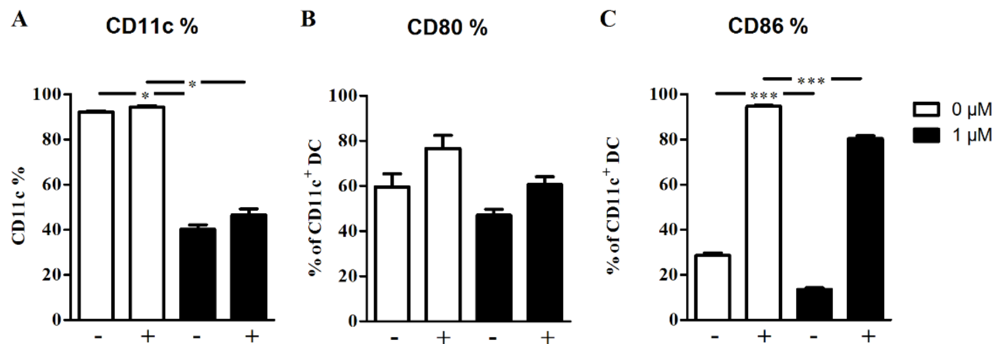

Figure 3.9. SAHA treatment of 10 days does not induce a permanent inhibition of CD80 or CD86 expression after SAHA removal. Primary immature DC were cultured out of mouse bone marrow in IMDM with GM-CSF and IL-4 for 10 days followed by a resting phase of 2d on culture medium without SAHA. Mature DC were generated by adding LPS for 24 hours before flow cytometric analysis of the respective molecules. A, Flow cytometry of CD11c expression on 10d SAHA-generated DC. B&C, Flow cytometry of CD80 and CD86 on 10d SAHA-treated DC. Results are shown as mean percentage ± SEM. n = 6, - or + is with or without LPS, * $p<0.05$, *** $p<0.001$.

3.4 Discussion

In this study we examined the ability of the histone deacetylase inhibitor SAHA to *ex vivo* generate tolerogenic CD11c$^+$ DC out of mouse bone marrow to suppress autoimmune responses in an *in vivo* mouse model of MS. We looked at the increased potency of SAHA to generate tolerogenic DC when treatment was started during the differentiation towards DC in comparison with the standard 24h treatment of already terminally differentiated DC. We showed that continuous SAHA treatment diminishes the number of newly generated CD11c$^+$ DC out of bone marrow. Furthermore, these SAHA-generated DC showed boosted characteristics of tolerogenic DC namely a reduced ability to respond to the TLR ligand LPS as evidenced by a reduced induction of MHC II, costimulatory molecules and proinflammatory cytokines in contrast to standard-treated DC. All these factors are essential features of immunostimulatory DC to drive T-cell priming and polarization towards Th1 and Th17 cells in autoimmunity and downregulation therefore suggests reduced ability to prime and induce T-cells. Although the SAHA-generated DC had tolerogenic properties *in vitro*, they were not able to fully counteract detrimental autoimmune responses when injected into an experimental model of MS. *In vitro* analysis of SAHA-generated DC showed that after removal of SAHA, costimulatory molecules were again upregulated in response to LPS. Our results demonstrate that testing the stability of tolerogenic phenotypes should be a priority when developing DC-based therapies to restore tolerance in the context of autoimmunity.

The mechanism behind the induction of the tolerogenic DC features by SAHA was related to SAHA's interference with STAT1 phosphorylation whereas STAT6 phosporylation was unchanged. STAT1 signaling is essential in the maturation process (269), in line with our observation of a complete block in upregulation of the costimulatory molecules CD80 and CD86 on immature DC upon LPS challenge. Moreover in our cell cultures, IL-4 and GM-CSF were used to generate CD11c$^+$ or conventional DC. A study of Jackson et al. proved that IL-4 and GM-CSF activate distinct and overlapping STAT pathways vital in DC differentiation and maturation. STAT1 phosphorylation and subsequently activation is detected in all stages of DC development. However, a more robust level is needed for complete maturation. In contrast to STAT1, STAT6 is only constitutively activated in immature DC (269). CCR5's ligand CCL5 is proven to be important in mediating leukocyte adhesion

towards the CNS in EAE (270, 271). Since SAHA-generated DC had a reduction in the chemokine receptor CCR5 one can expect that these tolerogenic DC are less capable to migrate to the CNS. FasL is involved in controlling cells by inducing apoptosis in autoreactive T cells. The observed reduction in FasL levels on SAHA-generated DC could thus interfere with the control of autoreactive T-cells. However in contrast to FasL, PD-L1 was not changed after 10d SAHA treatment. Expression by DC of co-inhibitory molecules such as PD-L1 is crucial for the downregulation of T-cell responses and the maintenance of immune homeostasis. It is described as one of the markers for tolerogenic DC (272, 273). Wölfle et al. showed that PD-L1 expression is regulated via STAT3 activation. Since our data showed no changes in PD-L1 levels, we can speculate that there was no interference on the STAT3 pathway as was also the case with STAT6. Other studies in DC revealed that STAT1 is involved in TLR4 signaling. This signaling leads to the induction of a range of genes involved in pro-inflammatory cytokines, chemokines and cell surface molecules (274-276). Our experiments showed that besides surface molecules also pro-inflammatory cytokine production (IL-6 and TNF-α) was strongly reduced when SAHA was present in the cultures. In contrast no changes in basal levels of IL-10 secretion were detected with or without SAHA. However we have to keep in mind that DC were stimulated with LPS which induces increased proinflammatory cytokine productions and is therefore not the appropriate stimulus for IL-10 induction. Although it can be concluded that treatment with SAHA on its own did not increase IL-10 production.
Taken together, SAHA at the start of the DC differentiation process induces phenotypic and functional changes that lead to immature CD11c$^+$ DC with tolerogenic features such as reduced antigen-uptake and presentation, low levels of costimulation and diminished cytokine production that can be explained by the observed reduction in TLR4 and STAT1 signaling. Still, other pathways,like NF-κB are also regulated by acetylation and hence may be blocked by SAHA (277).
When SAHA-generated tolerogenic DC were injected into EAE mice, we observed only a minor and temporal effect on the disease course. Interestingly, there was no additional clinical effect of SAHA pretreatment as untreated immature DC reduced the onset of clinical symptoms to the same extent. Moreover, a similar decrease of SAHA-generated and untreated immature MOG-DC was found at the level of anti-MOG T cell reactivity in secondary lymph nodes. The modest delay in symptoms can be explained by the immature DC phenotype which already

suggests intrinsic tolerogenic properties (149, 173). Tolerogenic characteristics induced by SAHA *in vitro* were shown to be only temporal since redrawal of SAHA overcame the block in maturation and restored the immunostimulatory characteristics *in vitro*. This is an experiment which is lacking in most studies which describe promising results when testing new tolerance inducing drugs *in vitro*. The instability of the SAHA-induced block in maturation explains why SAHA-generated DC are not able to limit autoimmune responses in EAE mice since they likely revert back to stimulating MOG-specific immune responses.

In summary, our work reveals that SAHA can have profound effects on DC differentiation leading to enhanced tolerogenic properties *in vitro* compared to the standard SAHA treatment on terminally differentiated DC. DC differentiated out of bone marrow in the presence of SAHA are hampered in their specific functions, such as antigen-uptake and presentation, providing costimulation and cytokines which are necessary for an optimal T cell proliferation and polarization. Although *in vitro* results were promising, a stable induction of tolerogenic DC to treat an autoimmune disease model could not be achieved with SAHA. Therefore our results imply that caution needs to be taken when working on DC-mediated tolerization therapies since *in vivo* stimuli can undermine the stability of tolerogenic DC leading to a lack of efficacy.

4

The DNA methylation inhibitor Decitabine completely blocks autoimmune mediated CNS demyelination

Based on:

TheDNA methylation inhibitor Decitabine completely blocks autoimmune mediated CNS demyelination.

Thewissen K[1], Hendriks JJA[1], Stinissen P[1], Slaets H[1], Hellings N[1]

Submitted

[1] Biomedical Research Institute, Hasselt University, Belgium

ABSTRACT

This chapter aimed to examine whether epigenetic treatment is able to significantly affect the development of autoimmune disease. We demonstrate that in vivo treatment with the DNA methylation inhibitor decitabine completely blocks the infiltration of pathogenic immune cells into the central nervous system and subsequent development of disease in experimental autoimmune encephalomyelitis, a preclinical model for multiple sclerosis. Further analyses of the immune response in primary and secondary lymphoid organs demonstrated that decitabine blocks priming of myelin-specific Th17 cells and significantly enhances the percentage of $CD4^{+}Foxp3^{+}$ regulatory T cells. These observations indicate that decitabine restores immune tolerance to self thereby leading to disease resistance. We conclude that decitabine is a powerful new therapeutic approach to consider for treating (auto)immune diseases with proven involvement of Th17 and Treg cells.

4.1 Introduction

DNA methylation is one of the main epigenetic markers. It is associated with transcriptional regulation and contributes to the development/polarization of a cell and its function (202, 278). Methylation takes place predominantly at cytosines that are followed by guanines (CpG) at so called CpG islands. This process is catalysed by DNA methyltransferases (DNMT) which transfers a methyl group to the 5‘-carbon of a cytosine. In normal cells DNA methylations are used to silence gene expression (199, 202, 278).

Recent evidence suggests that epigenetic changes are present in patients with multiple sclerosis (MS). MS is a chronic autoimmune disease of the central nervous system, characterized by demyelination, axonal degeneration and gliosis and is thought to develop in genetic susceptible individuals (14, 17, 279). Epigenetic changes could explain why some genetically susceptible persons remain healthy whereas others develop MS (199, 200, 205). Liggett and colleagues described significant differences in methylation patterns in 15 out of 56 investigated genes between healthy controls and MS patients. They were able to discriminate patients in remission from patients with a relapse based on the methylation status of the promoter (280) indicating that epigenetic changes may be directly involved in the progression of the disease course. Further evidence of the involvement of epigenetics in MS comes from the observation that epigenetic changes at specific loci are needed to establish a stable and specific differentiation to certain lineages of $CD4^+$ T cells (210, 211, 281). In this context, Janson et al. showed that the IL17a promoter region is hypomethylated in patients with MS. As a consequence an increased frequency of T cells can differentiate towards Th17 cells (212). Moreover, hypomethylation of the Treg-specific demethylated region (TSDR), a conserved CpG-rich island in the Foxp3 promoter region, is important for a stable and strong forkhead box protein 3 (Foxp3) expression. The continuous expression of Foxp3 is essential for the immunosuppressive function of regulatory T cells. Impairment of regulatory T cells have been reported in MS and other autoimmune diseases, consequently leading to expansion of autoreactive T cells that resist >Treg mediated immunosuppressive activity (83-85, 213, 226). Based on these observations, we hypothesize that the hypomethylating agent decitabine or 5-aza-2’-deoxycytidine (DAC) may be of therapeutic value in neuroinflammation by restoring regulatory T cell function.

DAC is an effective methylation inhibitor which induces hypomethylation by acting as a deoxyribonucleoside analogue. Hypomethylating agents do not target cells for immediate death, but cells are still able to undergo proliferation. In this manner, genes are reactivated that were previously methylation-silenced. DAC has been widely studied in cancer research (217-221) and is approved for the treatment of myelodysplasic syndrome (MDS) and acute myeloid leukemia (AML). In the following chapter we examine whether DAC has therapeutic potential in autoimmune disease using the MS animal model experimental autoimmune encephalomyelitis (EAE) to provide preclinical proof of concept. Moreover we examined the effect of DAC on the underlying autoimmune response, with special attention to $CD4^{+}Foxp3^{+}$ T cells and distinct pathogenic T cell subsets.

4.2 Materials and methods

4.2.1 Mice

Eight week old C57Bl/6J female mice were purchased from Harlan (Horst, the Netherlands). Animals were housed under standard conditions with water and food ad libitum in the animal facility of the Biomedical Research Institute of Hasselt University. The animals were allowed to acclimatize one week before the start of the experiments. Experiments were conducted in accordance with institutional guidelines and approved by the local Ethical Committee for Animal Experiments of Hasselt University according to European guidelines.

4.2.2 EAE induction and treatment with DAC

EAE was induced in ten week old female C57Bl/6J mice from Harlan using the Hooke Kit™ (Hooke Laboratories, Lawrence, USA) according to manufacturer's instructions. Briefly, mice were injected subcutaneously with 100 µl of 1 mg/ml myelin oligodendrocyte protein peptide $(MOG)_{35-55}$ emulsified in CFA containing 2 mg/ml mycobacterium tuberculosis H37Ra. Mice received an intraperitoneal injection of 100 µl of 2 µg/ml pertussis toxin directly after immunization and 24h later. Mice were intraperitoneally injected on a daily base with 0.15 mg/kg DAC dissolved in DMSO and further diluted to the indicated concentration in PBS (Sigma Aldrich, Bornem, Belgium) or equivalent of DMSO (Sigma Aldrich) starting at day 0 (start of EAE induction) until day 13. Mice were evaluated on a daily basis for changes in body weight and disease severity, using a standard 5-point scale: 0, no clinical symptoms; 1, decreased tail tone; 2, paresis of hind limbs; 3, paralysis of hind limbs; 4, quadraparesis; 5, death.

4.2.3 Determine frequency of immune cells

At day 9, 22 or 34 post induction, thymus, inguinal lymph nodes and spleens were isolated from EAE mice. Single-cell suspensions were obtained by passage through a 70 µM-mesh (BD). Spleen cells underwent a Ficoll (Histopaque-1077, Sigma Aldrich) density gradient centrifugation after which the buffy layer was collected and washed with PBS. Cell number was measured by an automated cellcounter (Scepter; Millipore, Overijse, Belgium). Fold changes of DMSO- or DAC-treated mice were calculated to healthy control mice.

4.2.4 MOG specific immune cell proliferation assay

Spleen cells were placed in RMPI-1640 (Lonza) medium containing 2% mouse serum (Harlan), 20 µM 2-mercaptoethanol (Sigma Aldrich), 1% non-essential amino acids (Sigma Aldrich), 1% sodium pyruvate (Sigma Aldrich) and 1% penicillin-streptomycin and restimulated with 20 µg/ml MOG. Unstimulated cells were used as a control. 5 µCi (^{3}H)-thymidine (Perkin-Elmer, Zaventem, Belgium) was added after 2 days of culture and cells were harvested 24h later using an automatic cell harvester (Pharmacia, Uppsala, Sweden). Radioactivity was quantified by means of a β-plate liquid scintillation counter (Perkin-Elmer). Stimulation indexes were calculated by dividing proliferation values in the MOG stimulated cultures by those in the respective non-stimulated controls.

4.2.5 Flow cytometry

An intracellular staining was done on cells isolated from the spleen, lymph nodes and thymus by first performing a surface staining with CD4-PERCP (BD Biosciences, Erembodegem, Belgium) for 30 min at 4°C. Cells were fixed with cytofix/cytoperm (BD cytofix/cytoperm plus; BD Biosciences) and incubated for 20 min at 4°C. Afterwards, cells were washed with 1xPerm/wash (BD cytofix/cytoperm plus; BD Biosciences) and incubated for 30 min at 4°C with the intracellular antibodies Foxp3-PE (BD Biosciences), IFNγ-APC (eBioscience, Vienna, Austria) or IL-17a-PE (eBioscience). In the case of an intracellular cytokine staining, cells were first stimulated with 25 ng/ml Phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich), 1 µg/ml Calcium Ionomycin (CaI; Sigma Aldrich) and Brefeldin A/Golgiplug (BD Biosciences) for 4 hours at 37°C and 5%CO_2. Unstimulated cells were used as a control. Data was acquired using the FACS Aria II (BD Biosciences) and analyzed with the FacsDiva software (BD Biosciences). As gating strategy, a gate was set on the $CD4^+$ population. Expression levels of surface molecules were analyzed within this CD4 gate.

4.2.6 Immunohistochemistry

Mice were perfused with ringer solution, containing 8.6 g NaCl, 0.3 g KCl, 0.33 g $CaCl_2$, 0.17 g $NaHCO_3$, 2.5 g $NaNO_2$, heparin and 10 mg phenolred. Spinal cords of C57Bl/6J mice were isolated and frozen into tissuetek at -80°C. A Leica CM3050S cryostat (Leica Microsystems, Diegem, Belgium) was used to cut frozen spinal cords into 10 µm sections. Immunohistochemistry was performed as

described previously (282). Briefly, sections were fixed for 10 min in acetone and blocked with Protein Block (Dakocytomation, Heverlee, Belgium) for 20 min. Primary antibodies were diluted in PBS and incubated overnight at 4°C. A rat anti-mouse CD3 antibody (1:100) was used to detect T cells and a rat anti-mouse F4/80 to stain macrophages (1:100; Both from AbD Serotec, Düsseldorf, Germany). As a seconday antibody a goat anti-rat alexa Fluor®555 (1:800; Life technologies, Merelbeke, Belgium) was used for 1 hour. Nuclear staining was performed during 10 min using 4.60-diamidino-2-phenylindole (DAPI; Life technologies). Sections were washed with PBS-0.05% Tween and cover slips were applied by using fluorescent mounting medium (Dakocytomation). Stained sections were visualized using the Nikon Eclipse 80i fluorescence microscope (Nikon, Kingston, UK) and quantified using NIS Elements BR4 software (Nikon).

4.2.7 Real-time PCR

Total RNA was isolated from blood mononuclear cells (PBMC) out of the spleens of mice using Rneasy mini kit (Qiagen, Venlo, The Netherlands) according to manufacturer's instructions. The NanoDrop 2000 spectrophotometer (Thermo Scientific, Erembodegem, Belgium) was used to determine the RNA concentration and quality. cDNA synthesis was conducted with qScript™ cDNA superMix (Quanta Biosciences, Gaithersburg, USA). The reverse transcription reaction was performed at 25°C for 5 min, 42°C for 30 min and 85°C for 5 min using the iCYCLER (Biorad, Nazareth-Eke, Belgium). RT-PCR was done on a steponeplus detection system (Applied Biosystems, California, USA). Universal cycling conditions were used: 95°C for 10 min, 40 cycles of 95°C for 15s and 60°C for 1 min. The reaction mixture contained 10 µM forward and reverse primers (see Table 4.1), SYBR green master mix (Applied Biosystems), RNase free water and 5 ng/µl cDNA template in a total reaction of 10 µl. The most stable reference genes were determined by GeNorm software as previously described (283). The most stable reference genes in PBMC isolated from spleen were HPRT, RPL13a and YWAZ. The comparative Ct method was used to obtain relative quantitations of gene expression. Lastly, the expression was normalized using the household genes and converted to fold change values using the 2-ΔΔCT method.

Table 4.1. Primer sequences for RT-PCR.

Gene	Primer: forward (FP) / reverse (RP)
Interleukin 1 bèta (IL1β)	FP:ACCCTGCAGCTGGAGAGTGT
	RP:TTGACTTCTATCTTGTTGAAGACAAACC
Interleukin 4 (IL-4)	FP:CTCACAGCAACGAAGAACACCA
	RP:AAGCCCGAAAGAGTCTCTGCA
Interleukin 6 (IL6)	FP:TGTCTATACCACTTCACAAGTCGGAG
	RP:GCACAACTCTTTTCTCATTTCCAC
Interleukin 10 (IL-10)	FP:AATAACTGCACCCACTTCCCA
	RP:CAGCTGGTCCTTTGTTTGAAAG
Interleukin 17 (IL17)	FP:ATCAGGACGCGCAAACATGA
	RP:TTGGACACGCTGAGCTTTGA
Interleukin 22 (IL-22)	FP:TTGAGGTGTCCAACTTCCAGCA
	RP:AGCCGGACGTCTGTGTTGTTA
Interferon gamma (IFNγ)	FP:TGAGGTCAACAACCCACAGGT
	RP:GACTCCTTTTCCGCTTCCTGAG
Tumor necrosis factor alpha (TNFα)	FP:CCAGACCCTCACACTCAG
	RP:CACTTGGTGGTTTGCTACGAC
Transforming growth factor beta (TGF-β)	FP:GGGCTACCATGCCAACTTCTG
	RP:GAGGGCAAGGACCTTGCTGTA
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	FP:GAGGTCACAAGGTCAAGGTG
	RP:GATTGACAGTGGCAGGCTTC
Inducible nitric oxide synthase (iNOS)	FP:GGCAGCCTGTGAGACCTTTG
	RP:GCATTGGAAGTGAAGCGTTTC
C-C Chemokine receptor 1 (CCR1)	FP:CAGAAACAAAGTCTGTGTGGACCAA
	RP:TGTGAAATCTGAAATCTCCATCCTT
C-C Chemokine receptor 2 (CCR2)	FP:CAGGTGACAGAGACTCTTGGAATG
	RP:GAACTTCTCTCCAACAAAGGCATAA
C-C Chemokine receptor 3 (CCR3)	FP:TTGAAGTGAGGTCTGAGCATCAA

	RP:AACGCATCACAGTTACAACATAATTCT
C-C Chemokine receptor 5 (CCR5)	FP:CAGGGCTGTGAGGCTCATCT
	RP:GGCAGCAGTGTGTCATTCCA
C-C Chemokine receptor 7 (CCR7)	FP:GTGGTGGCTCTCCTTGTCATT
	RP:TTCTTGGAGCACAAAGACTCG
C-X-C Chemokine receptor 2 (CXCR2)	FP:GAGAACCTGGAAATCAACAGTT
	RP:GTACCTGTGGCATGTACAATGG
CX3C Chemokine receptor 1a (CX3CR1a)	FP:TCAGCATCGACCGGTACCTT
	RP:CTGCACTGTCCGGTTGTTCAT
CX3C Chemokine receptor 1b (CX3CR1b)	FP:GCTCCGCAACTCGGAAGTC
	RP:AAGTAGCAAAAGCTCATGATAAGCAA
Chemokine (C-C motif) ligand 2 (CCL2)	FP:GGCTCAGCCAGATGCAGTTAA
	RP:AGCCTACTCATTGGGATCATCTT
Chemokine (C-C motif) ligand 4 (CCL4)	FP:GAAGCTTTGTGATGGATTACTATGAGA
	RP:GTCTGCCTCTTTTGGTCAGGAA
Chemokine (C-C motif) ligand 5 (CCL5)	FP:GGAGTATTTCTACACCAGCAGCAA
	RP:GCGGTTCCTTCGAGTGACA
Chemokine (C-X-C motif) ligand 1 (CXCL1)	FP:GCCTATCGCCAATGAGCTG
	RP:CTGAACCAAGGGAGCTTCAGG
Chemokine (C-X-C motif) ligand 2 (CXCL2)	FP:AACATCCAGAGCTTGAGTGTGA
	RP:TTCAGGGTCAAGGCAAACTT
Chemokine (C-X-C motif) ligand 5 (CXCL5)	FP:GCTGCCCCTTCCTCAGTCAT
	RP:CACCGTAGGGCACTGTGGAC
Hypoxanthine-guanine phosphoribosyltransferase (HPRT)	FP:CTCATGGACTGATTATGGACAGGAC RP:GCAGGTCAGCAAAGAACTTATAGCC
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHAZ)	FP:GCAACGATGTACTGTCTCTTTTGG RP:GTCCACAATTCCTTTCTTGTCATC
60S ribosomal protein L13a	FP:GGATCCCTCCACCCTATGACA
	RP:CTGGTACTTCCACCCGACCTC

4.2.8 Statistics

Data were statistically analyzed using GraphPad (Software Prism, La Jolla, CA, USA) and are presented as mean ± SEM. Normality was checked with a D'Agostino & Pearson omnibus normality test. One-way ANOVA followed by a Tukey post-test for testing individual groups were performed. For EAE experiments a two-way ANOVA was used dependent on time and treatment followed by a Bonferroni test to examine significance between treatment groups and the control DMSO group. Results were was considered significant when p values were <0.05.

4.3 Results

4.3.1 DAC inhibits the development of EAE

We first investigated if DAC had an effect on the development of EAE by treating mice with 0.15 mg/kg DAC (n=10) during two weeks starting from the day of EAE induction. Control EAE mice received equivalent injection of vehicle (0.3% DMSO, n=10). Mice treated with DAC did not develop any clinical symptoms of EAE (Fig. 4.1a). Moreover, DAC-treated animals did not show the characteristic loss in weight when EAE develops, as can be seen in the control EAE group (Fig. 4.1b). Next, we analysed immune cell infiltration in the spinal cords of EAE mice at 34 days post immunization (dpi). Significant T cell and macrophage infiltration was detected in the spinal cord of vehicle treated EAE mice, while no immune cells were present in the CNS of DAC-treated mice (Fig. 4.1c-h).

These results demonstrate that DAC is capable of counteracting the development of EAE by inhibiting the infiltration of pathogenic cells.

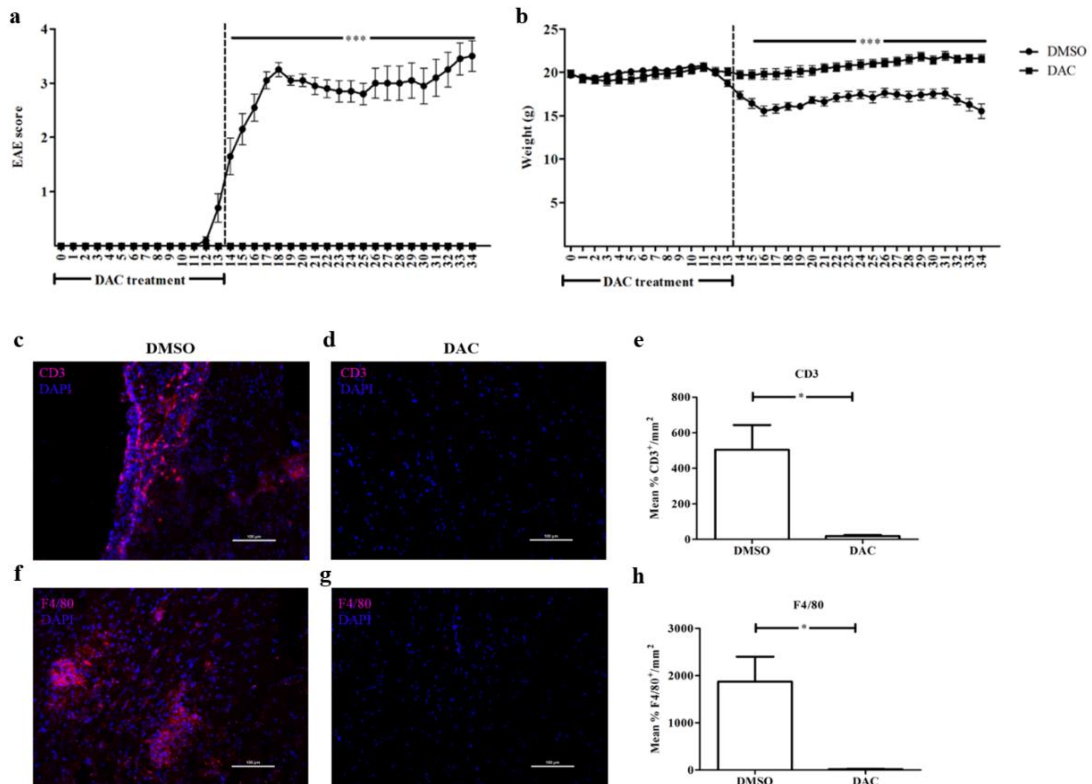

Figure 4.1. DAC treatment inhibits the development of EAE. Clinical score (a) and weight (b) of mice that were injected intraperitoneally at a daily base with 0.15 mg/kg DAC or equivalent of DMSO during the first two weeks of EAE (until the dashed line, 10 mice/group). c-h Immunohistochemical analyses of spinal cord tissue from EAE mice treated with DMSO (c,f) or DAC (d,g) 34 dpi, stained with CD3 (c-e) or F4/80 antibodies (f-h). 5 representative sections were stained for each mouse. n = 5, * $p<0.05$.

4.3.2 DAC treatment reduces the number of immune cells present in primary and secondary lymphoid organs and inhibits priming of MOG reactive T cells

Because mice did not develop EAE after DAC treatment, we next investigated if DAC affects immune cell numbers and their phenotype in primary (thymus) and secondary lymphoid organs (lymph nodes and spleen) during the induction (9 dpi) and chronic disease phase (22 dpi). Fold changes of cell numbers of DMSO- or DAC-treated mice were calculated relative to healthy control mice. The number of cells in the thymus at 9 dpi does not differ between EAE and healthy control (HC)

mice. However DAC treatment significantly reduced immune cell numbers in the thymus compared with DMSO-treated EAE mice and HC (Fig. 4.2a). Based on the important role of the thymus in T cell development, it can be speculated that DAC interferes with this process.

In lymph node and spleen, DMSO-treated EAE mice showed an increased number of cells compared to HC, reflecting the ongoing autoimmune response. This increase was not detected in DAC-treated EAE mice (Fig. 4.2b-c). To test whether MOG-specific responses are modulated by DAC treatment, spleens were collected when the immune response is at its maximum in secondary lymphoid organs (9 dpi) and during the chronic phase (34 dpi). DAC-treated EAE mice had a significant reduction of MOG-specific cell responses at 9 dpi in comparison with DMSO injected EAE mice (Fig. 4.2g). During the chronic phase low levels of MOG responses were measured in both groups, reflecting diminished inflammation during the chronic phase of EAE (Fig. 4.2h), and no significant differences were detected between the groups (Fig. 4.2d-f)

These observations show that the thymus, an important primary lymphoid organ for T cell development is affected by DAC treatment. Moreover, the increase in cells in the lymph nodes and spleen, reflecting a MOG-specific immune response during EAE, was absent in DAC-treated EAE mice.

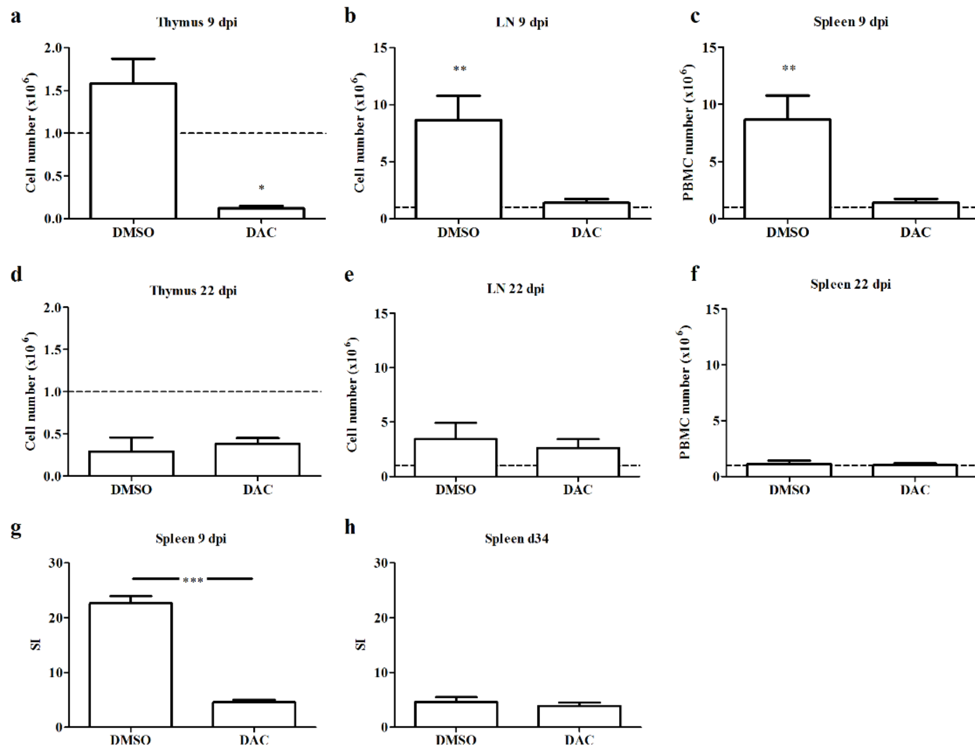

Figure 4.2. No increases in immune cell numbers are detected in lymph nodes or spleens of DAC treated mice, whereas the cell frequency in the thymus is reduced compared to DMSO controls. a-c**,** Fold changes of frequency of cells from DMSO or DAC treated mice isolated out of the thymus (a), Lymph nodes (b) or spleen (c) at 9 dpi compared to HC. d-f, Fold changes of frequency of cells from DMSO or DAC treated mice isolated out of the thymus (d), lymph nodes (e) or spleen (f) at 22 dpi compared to HC. Results are shown as mean percentage ± SEM. n = 3-6, * p<0.05, ** p<0.01. g, Stimulation index of MOG responses of spleen cells ((^{3}H)-thymidine labeled) isolated on day 9 out of mice injected with DAC or DMSO. h, Stimulation index of MOG responses of spleen cells ((^{3}H)-thymidine labeled) isolated on 34 dpi out of mice injected with DAC or DMSO. Results are shown as mean weight, EAE score or SI ± SEM. n = 4-5, dpi = days post immunization, SI = Stimulation Index, *** p<0.001.

4.3.3 DAC treatment reduces the number of Th17 cells in the spleen of EAE mice

Next, we examined if DAC inhibited MOG-specific responses by modulation of Th cell differentiation. Hereto percentages of Th1 and Th17 cells were quantified in

lymph nodes and spleen of DMSO- and DAC-treated EAE mice at 9 dpi. The percentage of Th1 cells in spleens was not affected by DAC treatment (Fig 4.3a). In contrast, the percentage of Th17 cells was significantly decreased in spleen after DAC treatment compared to vehicle treated EAE mice (Fig. 4.3b). No significant differences in Th1 and Th17 cells were detected in lymph nodes (Fig. 4.3c-d).

These results suggest that differentiation towards Th17 cells is inhibited by DAC treatment, resulting in a lower level of Th17 cells in spleens of EAE animals.

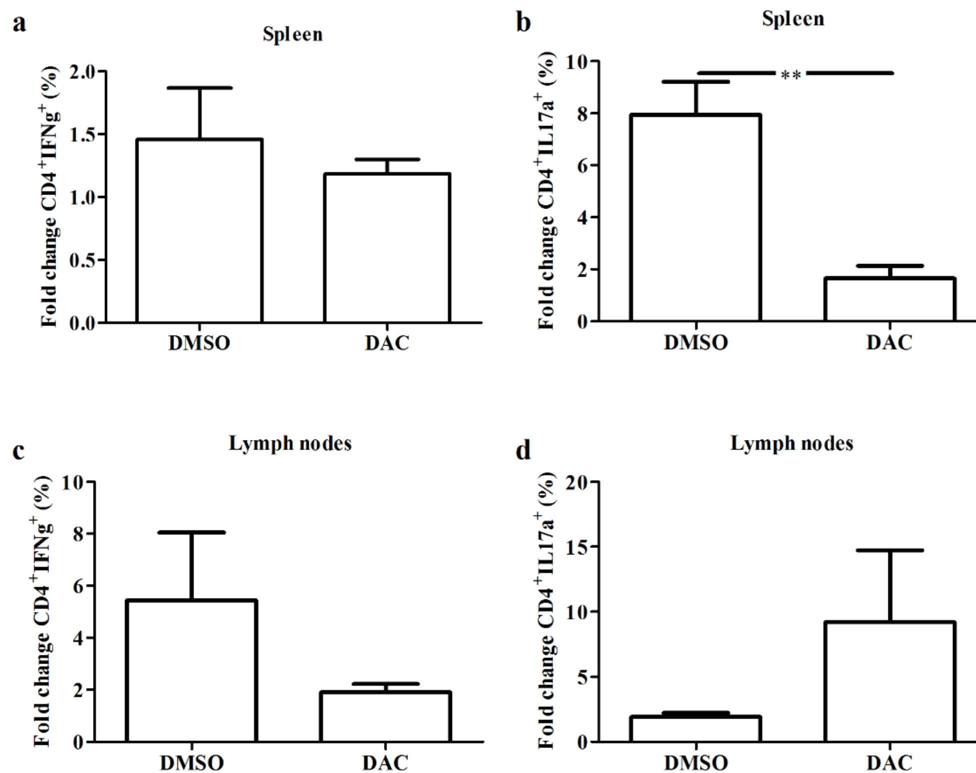

Figure 4.3. DAC treatment reduces the presence of Th17 cells in the spleen. Flow cytometric analysis of IFNγ (a) and IL-17a (b) expression on $CD4^+$ T cells in spleens of DAC and DMSO treated animals at day 9. Flow cytometric analysis of IFNγ (c) and IL-17a (d) expression on $CD4^+$ T cells in lymph nodes of DAC and DMSO treated animals at day 9. Results are shown as mean percentage ± SEM. n = 4-5, ** $p<0.01$.

4.3.4 Foxp3 expression is increased by DAC in $CD4^+$ T cells

The DNA methylation status of specific CpG islands in the Foxp3 promoter region, called the Treg-specific demethylated region (TSDR), is important for a stable

expression of Foxp3. Demethylations at this site can discriminate regulatory T cells from conventional $CD4^+$ T cells (284, 285). Therefore, we investigated whether DAC treatment increased Foxp3 expression in the $CD4^+$ T cell population in the thymus and spleens of EAE mice. At day 9 post EAE induction, $CD4^+$ T cells isolated from DAC-treated mice showed a significant increase in $CD4^+Foxp3^+$ cells in the thymus and spleen compared to DMSO-treated EAE mice (Fig. 4.4a-b). On 22 dpi (9 days without DAC treatment), $CD4^+Foxp3^+$ cell in DAC-treated mice were back to control levels and no significant differences were detected (Fig.4. 4c).

Thus, besides suppressing the induction of Th17 cells, DAC treatment induces regulatory T cells which contribute to the therapeutic effect of DAC.

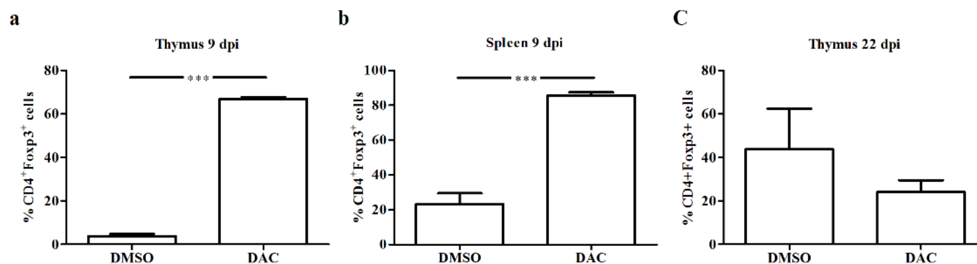

Figure 4.4. Foxp3 expression is increased in $CD4^+$ T cells during DAC treatment. Flow cytometric analysis of Foxp3 expression on $CD4^+$ T cells in thymus (a) and spleens (b) of DAC and DMSO treated animals at day 9. c, Flow cytometric analysis of Foxp3 expression on $CD4^+$ T cells in thymus of DAC and DMSO treated animals at day 22. Results are shown as mean percentage ± SEM. n = 3-5, dpi = days post immunization *** $p<0.001$.

4.3.5 DAC reduces the upregulation of genes important in driving inflammation

To elucidate which cellular pathways are influenced by DAC, we performed RT-PCR on PBMC of spleens derived at 9 dpi from DAC or DMSO treated EAE animals. We tested the effect of DAC on the expression of genes which are categorized into pro- and anti-inflammatory cytokines, chemokines and chemokine receptors. Genes such as TNF-α, TGF-β and IL-10 were not affected by DAC treatment (Fig. 4.5a). However cytokines specifically involved in the differentiation of T cells towards Th17 cells were reduced after DAC treatment (IL-6, IL-1β) as were cytokines produced by Th17 cells (IL-17, IL-22, GM-CSF, Fig. 5a). Reduced levels of IL-4, IFNγ and iNOS were also found in DAC treated mice compared to DMSO

treated mice (Fig. 4.5a). The level of gene expression of chemokines and chemokine receptors important for the recruitment of immune cells to the CNS, are reduced after DAC treatment with the exception of CCL5 which is evenly expressed in both groups (Fig. 4.5b). Expression of CCR7, a migration molecule controlling homing towards secondary lymphoid organs is increased in DAC treated mice (Fig. 4.5b).

From these data, we can conclude that genes involved in driving inflammation by means of inducing T cell differentiation (IL-6, GM-CSF, IL-1β) or migration towards inflammatory sites (CCR1, CCR2, CCR5, CCL2, CCL4, CCL5) are downregulated by DAC treatment. This combined with the increase of $CD4^{+}Foxp3^{+}$ regulatory T cells thus may result in the inhibition of the autoimmune response seen in DAC treated EAE mice.

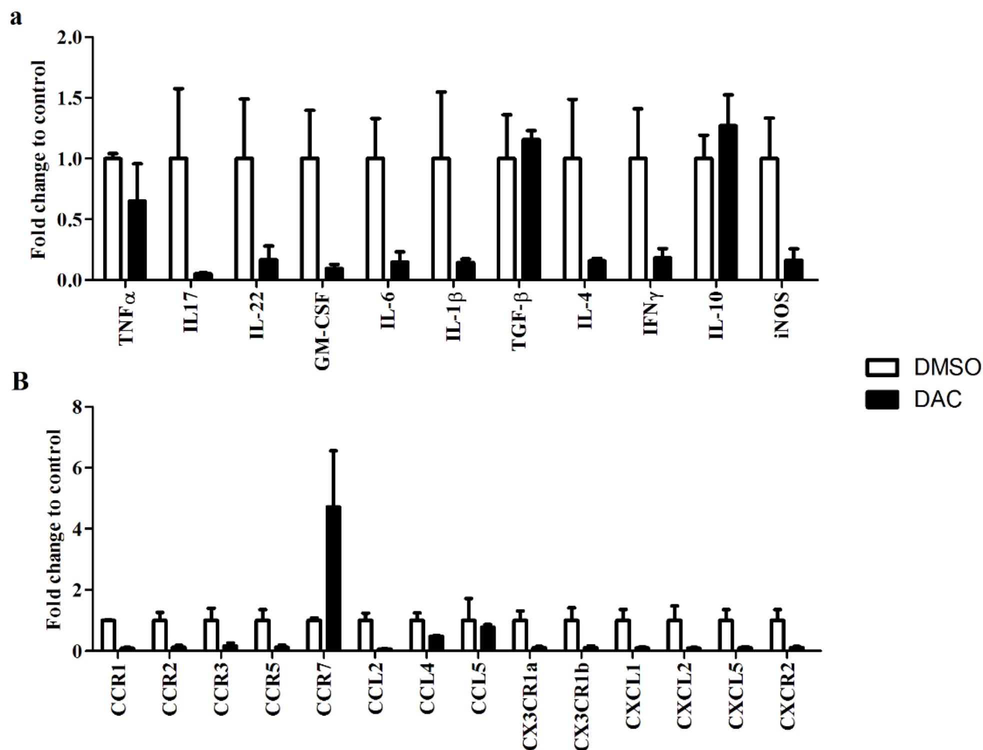

Figure 4.5. DAC reduces the upregulation of genes involved in driving inflammation and migration. Data on the expression of cytokines (a), chemokines (b) and chemokine receptors (b) measured with RT-PCR on PBMC from spleens of DAC and DMSO treated mice at d9. The expression was normalized using the most stable housekeeping genes (HPRT,

RPL13a and YWHAZ) and converted to fold change values using the $2^{-\Delta\Delta CT}$ method. Fold changes are calculated to control with DMSO treated animals as controls. N=2.

4.4 Discussion

In this study we show that DAC-treated mice do not develop any symptoms characteristic of EAE. The resistance to develop EAE was associated with reduced MOG-reactive responses in the spleen and a block of infiltration of macrophages and T cells into the CNS. Mice were followed up until 20 days after the stop of DAC treatment and were still clear of any symptoms, suggesting an induction of tolerance against MOG. Studies in models of asthma and diabetes also showed therapeutic potential of DAC (226, 286). Here we show for the first time beneficial effects in EAE which further supports the use of DAC and associated pathways as an potential strategy for the treatment of autoimmune disease (226, 286).

Since DAC treatment blocked the development of EAE in mice, we next investigated what the effect of DAC was on important primary (thymus) and secondary lymphoid organs (inguinale lymph nodes and spleen). Cell numbers into the lymph nodes and spleen were increased in DMSO treated mice whereas levels were similar between DAC treated mice and healthy controls. This accumulation of cells in secondary lymphoid organs is a key characteristic of the ongoing autoimmune response and is resolved after the mice progress into a chronic phase with less emphasis on inflammation (120, 287). Most interesting was the finding that the number of immune cells in the thymus was clearly reduced after DAC treatment. Due to the important role of the thymus in the development of T cells, it can be speculated that a shrinkage of the thymus leads to a reduced thymic output of T cells in comparison with DMSO-treated EAE mice and healthy control mice. This effect on thymic output can be translated to reduced populations of effector T cells in the periphery and hence the observed clinical effect. In that regard we detected reduced MOG-responses in the spleens of DAC-treated EAE mice. Furthermore, we showed that Th17 cells were reduced in the spleens after DAC treatment. Besides thymic atrophy during the acute phase of EAE, Foxp3 expression is also increased in the thymus as well as in the spleen, indicating an induction of regulatory T cells. This Foxp3 expression regained normal levels after treatment was stopped, suggesting a temporary boost of regulatory cells. However, after treatment mice did not develop EAE signifying the role of DAC in establishing a balance to tolerance of the immune response. Zheng and colleagues proved that regulatory T cells induced by DAC from thymus and spleen had a higher immunosuppressive capacity (226). Other studies showed

that DAC regulates Foxp3 expression in human T cells by DNA methylation leading to functional and stable Treg (213). In contrast, a recent human study with DAC done by Kehrmann et al., demonstrated that these DAC induced Tregs do not suppress the proliferation of responder cells. Furthermore, they did not find any relevant hypomethylations within the TSDR or expression of Treg specific genes (288). Due to this controversy, it remains to be investigated whether DAC leads to a stable induction of the regulatory phenotype and which precise pathways are involved in the observed effect.

Previously studies on Foxp3 expression reported that the DNA methylation status in the TSDR region is important for a stable expression of Foxp3 (213, 284, 285). Hypomethylation in this region induced by DAC leads to an upregulation of Foxp3 and hence the presence of $CD4^+$ T cells with a regulatory phenotype. It is known that transcription factors involved in Th17 cells and adaptive Tregs are interconnected (76, 289). In the presence of TGF-β a naïve T cell can become a regulatory T cell by expressing Foxp3 upon TCR stimulation (290). When an immune response is going on and inflammatory stimuli are present, more specifically IL-6, this cytokine acts in concert with TGF-β to upregulate RORγt expression. This transcription factor is crucial for the development of Th17 cells and blocks the differentiation of regulatory T cells (291-293). The opposite is seen when Foxp3 is active. This interplay between Treg and Th17 cells is important to keep the immune system in balance to protect against autoimmunity and respond accurately during inflammation. In our study, mRNA data in spleen cells showed that expression of TGF-β and IL-10 remained high whereas other pro-inflammatory cytokines are significantly reduced after DAC treatment. We showed that Th17 cells were reduced in the spleen and mRNA data confirmed the absence of the Th17 cytokines IL-17, IL-22 and GM-CSF. Moreover Th17 polarizing cytokines such as IL-6 and IL-1β were clearly reduced (75, 289, 294, 295). Taken together with the role of TGF-β and Foxp3 in inducing regulatory T cells, DAC interferes with the differentiation towards Th17 cells whereas Th1 cells are not affected. The reduced expression of chemokines and associated receptors together with the lack of immune cells in the CNS suggests that DAC indeed blocks migration into the CNS. However migration towards lymphoid organs is increased as CCR7 mRNA expression is high in PBMC of the spleen. Newly generated naive cells out of the thymus are more prone to home to the spleen and lymph nodes were they are subjected to regulation by Foxp3 expressing T cells induced by DAC.

This regulation in combination with the observed migration block is likely to explain why DAC treated mice did not show any clinical symptoms.

In summary, our data demonstrate that DAC treatment efficiently counteracts the development of EAE by blocking the infiltration of pathogenic macrophages and T cells. Moreover DAC increased the presence of $CD4^{+}Foxp3^{+}$ T cells and diminished the presence of typical pathogenic Th17 cells. These results support the potential of DAC to be explored as a possible new therapeutic approach to treat (auto)immune diseases where Th17 and/or Treg have proven to contribute to the pathogenesis.

5

Summary and general discussion

5 Summary and general discussion

MS is a complex disease with a lot of contributing factors. Although the etiology behind the disease is unknown, it is very clear that the immune system is dysregulated. In this autoimmune disease, activated autoreactive T cells infiltrate the CNS and initiate an inflammatory response. These T cells are able to escape central tolerance that normally occurs in the thymus (3). Autoreactive T cells are also found in healthy individuals, but do not lead to the development of autoimmunity (296). This suggests the presence of mechanisms that counteract the activation and expansion of self-reactive T cells (297). Studies have shown that Treg may play an important role herein. For example, $CD4^{+}CD25^{+}$ Tregs are less functional in MS patients (83-85, 87). To date it is not yet known what causes the reduced presence and function of natural $CD4^{+}CD25^{+}$ Treg in MS patients. The recently identified susceptibility genes for MS indicate a possible genetic influence on Treg dysfunction (27, 298, 299). Another possible reason is the depletion of Treg due to exhaustion after chronic inflammation (300, 301). Recently, it was shown that DC play a role in the homeostasis of Treg (234, 302-304). The group of Darrasse observed that a loss of DC leads to a reduction in Treg. This DC-dependent loss of Treg is accompanied by an increase in the number of auto-aggressive T cells and the production of pro-inflammatory cytokines in mice (234). In this thesis we further elaborated on the role of DC in MS by determining if circulating DC subsets undergo changes in phenotypical, functional and migratory profiles in an extensive cohort of MS patients during different disease stages. Furthermore, we evaluated whether DC targeting or epigenetic treatment is able to restore the immune balance in the context of MS. In the following paragraphs, the main results are described and discussed.

5.1 Aim I: Characterization of the DC compartment in MS patients

Because of their unique capacity to shape immune responses, DC are thought to play a pivotal role in the immunopathogenesis of several autoimmune disorders, including MS. The extreme low frequency of circulating DC and lack of access to lymphoid tissues makes it difficult to study the DC compartment in humans. In the following paragraphs, our data are integrated in existing literature on peripheral blood, CSF or post-mortem material to gain an overview of the exact role of the DC compartment in MS (figure 5.1).

Do circulating DC subsets have a more stimulating or regulating character?

In general, human DC studies demonstrate that both DC subsets, cDC and pDC, are altered in the peripheral blood of MS patients and more prone to drive a proinflammatory T cell response (176-180). Karni and colleagues showed that cDC in the blood of MS patients had an increased expression of CD40 and CD80. In RRMS patients the production of pro-and anti-inflammatory cytokines were still balanced, but the cytokine profiles of cDC shifted towards pro-inflammatory (IL-12, TNFα and IFN-γ) in the progressive state of MS (176). Accompanied by the enhanced activation state of cDC is a rise in pro-inflammatory T cell responses (181). Moreover, DC are the only cells besides activated macrophages capable of secreting IL-23. This cytokine together with IL-6 and IL-1β promotes the differentiation of naïve T cells into IL-17 producing Th17 cells. Th17 cells have shown to play an essential role in MS and IL-17 is capable of disrupting the BBB.

In **chapter 2** we reexamined the circulating DC subsets in a large cohort of MS patients taking into account the influence of medication as well as the clinical phase of MS patients. We confirmed that cDCs of RRMS patients are in a more immunogenic state represented by a higher expression of the costimulatory molecule CD86 and increased production of IL-12p70. The view that cDC are in a more immunogenic or activated state in MS is not surprising because these are the conventional inducers of primary T cell responses (149, 158).

However, some studies show conflicting results on the functionality of circulating DC in MS patients (178, 236). For example a study done by Stasiolek demonstrated that circulating pDC present a rather immature expression profile of costimulatory molecules (CD40, CD80 and CD83) in contrast to the above statement. The potency of pDC to generate regulatory T cells was hampered (178). In contrast, pDC are inefficient in priming T cells and rather induce T cell anergy and support the development of regulatory T cells in the steady-state. Secretion of IFN-α by pDC shapes the immune response by influencing other regulatory cells including cDC and IFN therapy has proven beneficial for MS patients (156, 157, 161-163). Although our results do not detect increased costimulatory molecules on pDC of MS patients, a significant lower baseline level of IFN-α was present in MS patients independent of disease stage compared to healthy controls. This indicates that pDC may be hampered in their regulatory function. Failure of regulatory mechanisms such as pDC, leads to progression of

inflammation. Indeed in EAE, depletion of pDC results in exacerbations of disease severity, enhanced $CD4^{+}$ T cell activation and increased production of IL-17 and IFN-γ (305). In accordance, Karni et al. showed that in RRMS there is an increased production of pro- and anti-inflammatory cytokines and this balance between pro and anti-inflammatory cytokines is gone when patients go into the chronic phase of MS and produce only high amounts of pro-inflammatory cytokines. Our study together with others demonstrate that pDC have an essential role in negatively regulating pathogenic T cell responses (178, 305).

To summarize, our data gives evidence to a dual role of circulating DC in the pathogenesis of MS. Conventional cDC are in a more immunogenic state in MS and drive/support the immune responses that lead to damage in the CNS. In contrast, the role of pDC is different. pDC are known to regulate immune responses, but in MS patients we suggest that these cells have a diminished regulatory capacity as was indicated by a lower production of IFN-α. The different roles of DC subtypes in an immune response can open new therapeutic strategies, like for example boosting regulatory pDC in counteracting an autoimmune reaction.

Do circulating DC subsets differ in frequencies or migration patterns between MS patients and healthy individuals?

DC determine the profile of immune responses depending on the co-stimulation they provide. Alterations in the DC compartment can lead to the induction, perpetuation or aggravation of inflammation. In **chapter 2** we found a lower frequency of circulating pDC in the peripheral blood of CPMS patients whereas no differences in absolute numbers of cDC were detected. While other studies do not observe differences in pDC in RRMS patients, we detected altered frequencies in CPMS patients, indicating a possible role of pDC in the progression of MS.

Furthermore, DC are capable of migrating into the parenchyma and to take up myelin. Serafini et al. identified DC in the perivascular cuffs of early and chronic MS lesions in post-mortem brain tissue. These DC contained components of myelin and cells expressing mature DC markers such as CD83 were found. Some of the perivascular DC were detected in close proximity to proliferating lymphocytes (153). In **chapter 2** we showed that the migration-associated marker CCR5 was increased on cDC and pDC of respectively RRMS and CPMS patients compared to HC. Subsequent in vitro migration assays revealed that both subsets are more

prone to migrate towards RANTES and MIP-1β respectively. Because these ligands are also increased in MS lesions (242, 243), our migration assays provide indirect evidence that circulating DC subsets of MS patients can indeed migrate towards the CNS and draining lymph nodes. As CCR5 was increased on cDC in RRMS patients, this suggests an important role of cDC in perpetuating the damage in the acute phase of MS. After uptake of myelin components, DC migrate to the deep cervical lymph nodes were they activate T cells or reside locally to expand T cells in the CNS. This process can break the immune tolerance towards CNS antigens and initiate or enhance the damage in the CNS (179). Moreover, flow cytometric analysis of circulating DC revealed a significant higher expression of CCR5 and CCR7 on pDC. Our study confirmed that pDC of MS patients have a higher potency to migrate to CCL19, CCL21, RANTES and MIP-1β than those of healthy individuals. Taken together our data suggest an increased pDC migration to the CNS and lymph nodes, more specifically to the T cell zones. The increased migratory capacity of pDC can explain the reduced frequency of pDC in the peripheral blood of MS patients. pDC are migrating with a higher rate to the CNS to regulate immune responses. However due to their reduced regulatory character in MS, they fail in their task.

How can genetic variations impact the DC compartment in the context of MS?

Our research group revealed for the first time differences in DC subsets when MS patients are stratified based on the presence of the susceptibility genes HLA-DRB1*1501 or IL7-Rα. It was shown that HLA-DRB1*1501 has a high affinity in binding MBP. This HLA affinity directly determines the immunodominance in a cellular immune response. Moreover, the group of Vergelli revealed that binding to HLA-DR prevents the proteolytic cleavage of the immunodominant middle region of MBP by the endosomal protease cathepsin D (306). This makes the peptide ideally available for T cell recognition. MS patients that are HLA-DRB1*1501 carriers have reduced numbers of cDC circulating in the peripheral blood. The complex between for example MBP(84-102) and HLA-DRB1 molecules is more immunogenic for MBP-reactive T cells and since this susceptibility gene is associated with more focal abnormalities in the spinal cord and a more severe disease course (71, 247, 248), the decreased cDC frequency in this genetically predisposed MS population may indicate increased migration towards lymph nodes

or CNS. It was proven that the frequency of cDC was increased in the CSF of MS patients, suggesting that cDC are present to boost local immune responses (177, 307). This is in line with our findings of increased expression of inflammatory chemokine receptors. In addition, pDC are decreased in the blood of MS patients not carrying the protective haplotype 2 IL7Rα allele. IL7Rα chain combines with the thymic stromal lymphopoietin receptor (TSLPR) on DC to bind respectively IL-7 or TSLP. IL-7 signaling is essential for central T cell development and homeostasis of the peripheral T cell pool, whereas TSLP plays a role in the DC-mediated differentiation of Foxp3^{+} regulatory T cells in the thymus and promotion of T helper type 2 differentiation in the periphery. Since DC are involved in antigen presentation and T cell polarization, the above-mentioned MS-associated risk genes may influence the interaction between DC and T cells. (237-239, 308-311). Donors not carrying haplotype 2 of IL7Rα, have a SNP in exon 6 where the T base is replaced by a C base resulting in increased skipping of exon 6. This skipping of exon 6 leads to production of the soluble form of IL-7Rα (25, 238, 239, 311). Since DC play an important role in T polarization, subtle changes in the balance between the soluble and membrane-bound form of IL-7Rα is expected to affect T cell functionality. In this light haplotype 2 is thought to be protective in MS, since a higher level of membrane bound IL-7Rα results in moreavailability of functional receptor molecules to bind IL-7 or TSLP. In MS patients not carrying the protective haplotype 2, the TSLPR may be decreased because of the lower level of membrane bound IL7-Rα hampering the efficiency of pDC in inducing regulation and enhancing the request of more functional pDC in target tissues. Although the exact levels of TSLP and the TSLPR in MS patients is unclear, more research in the field of susceptibility genes and associated molecules can give a better overview of the immune imbalance in specific MS subpopulations. Therefore future experiments include functional assays on DC, macrophages, B and T cells isolated from genetically predisposed MS patients to unveil the exact immune imbalance leading to better fine-tuning of a patient specific therapy.

Our observations illustrate the importance of studying different DC subpopulations in the context of MS. Each DC subset has its own tasks that may be disturbed in autoimmunity leading to a patient group with a widespread range of symptoms and patients that show a rapid progression in comparison with others. In addition, susceptibility genes and associated molecules can superimpose more

heterogeneity between MS patients, causing the complexity of MS. As a consequence certain patients will benefit from a particular therapy whereas other patient subpopulations will not. Therefore, it is important to further investigate the different immune components and associated genetic factors to gain a more detailed view of each MS subpopulation with the goal of fine tuning prognosis and therapies.

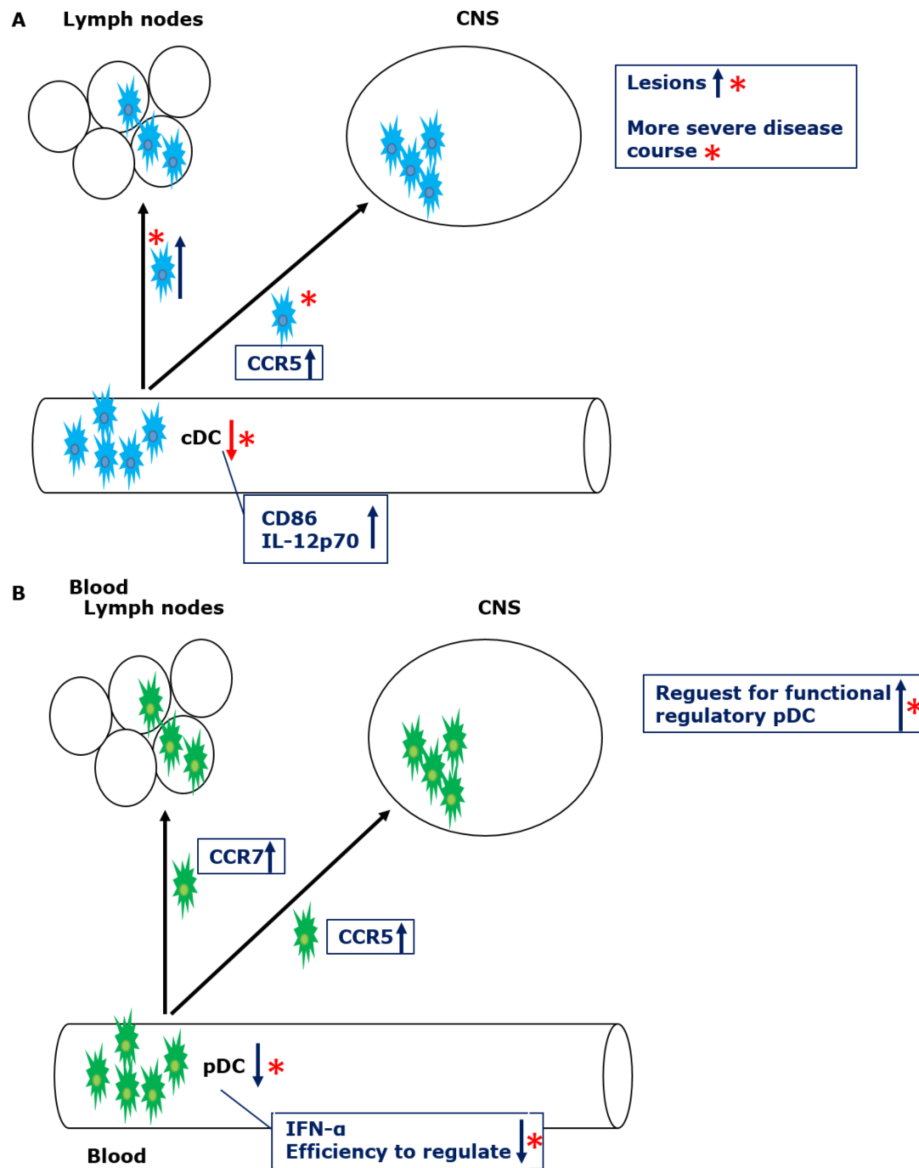

Figure 5.1. Summary of the most important alterations in the DC compartment of MS patients as reported in this thesis. A. cDC show a higher immunogenic and activated state in MS patients (CD86, IL-12p70) and migration assays suggest an increased migration towards the CNS (CCR5) and Lymph nodes. Changes (red arrows) or a possible influence (red asterisks) of HLA-DRB1*1501 are indicated. B. pDC of MS patients have a diminished regulatory character and show an increased migration towards lymph nodes and CNS (CCR7, CCR5). Suggested influences (red asterisks) of IL7Rα non haplotype 2 are indicated.

5.2 Aim II: Generation of tolerogenic DC by the histone deacetylase inhibitor SAHA to restore the immune tolerance in EAE

Studies have shown that DNA methylations and histone modifications can regulate the expression of genes associated with the immune system, thereby modifying the development of innate and adaptive immune responses. Manipulation of these epigenetic mechanisms can possibly modulate the immune response and lead to new therapeutic strategies. In **chapter 3** we examined whether the histone deacetylase inhibitor SAHA can generate tolerogenic DC. However several general parameters must be met and optimized before a DC therapy can be approved to safely treat patients.

Which parameters are important when making a tolerogenic DC-based therapy?

Firstly, a sufficient number of DC needs to be obtained. In humans, peripheral blood has a low DC number, ranging from 0.09 to 0.42% of the leukocyte population. This population is insufficient to obtain significant numbers to make a DC based cell product. Alternatively, myeloid DC can be easily generated *ex vivo* from isolated monocytes or $CD34^+$ precursor cells using stimuli such as GM-CSF and IL-4 (312-314). Secondly, generated DC have to be efficiently loaded with antigens to induce an antigen specific reaction in vivo and to reduce the occurrence of non-specific and detrimental immune responses. Third interest is the route of DC delivery. It has been suggested that intravenously injected cells rather lead to tolerogenic effects, whereas subcutaneous injections lead to immunogenic reactions. New studies indicate that subcutaneous injections can also induce tolerance. Other injection routes frequently used in DC research are dermal injections or even administration of DC directly in draining lymph nodes. In this last method, the migration problem of DC out of the skin is circumvented. However the intranodal injections can lead to the disruption of the lymph node and cells need to travel to adjacent lymphoid structures (174, 193, 195, 313). Each method of administration has it benefits and depending on the disease in question a different approach can be preferred. Besides basic parameters, such as DC generation, antigen loading and administration, the most important parameter is the generation of DC that have a stable tolerogenic phenotype also *in vivo*.

Can epigenetic treatment lead to the generation of stable tolerogenic DC?

To generate stable tolerogenic DC several time points in the life of a DC can be targeted. By using substances that interfere with their differentiation, antigen uptake, maturation, migration and function. We investigated the influence of the HDAC inhibitor SAHA on the differentiation/maturation of *in vitro* generated cDC. We hypothesized that the potency of SAHA to generate tolerogenic DC can be boosted when DC are continuously treated during their differentiation from bone marrow cells (10d treatment) compared to the standard 24h treatment and that these SAHA-generated DC have therapeutic potential in autoimmune disease. In **chapter 3** we showed that 10d SAHA treatment affects the cDC at several points. Firstly, differentiation of cDC from bone marrow progenitor cells are hampered as was clear from the reduction in $CD11c^{+}$ DC. The progenitor cells that are capable to differentiate towards $CD11c^{+}$ DC are maturation resistant when SAHA is present. When LPS was added to induce maturation and activation of DC, costimulatory expression remained at a low level. This was an indication that treated DC remain in an immature resting phase even after an activation stimulus. An immature state is more associated with tolerogenic reactions, because in the steady-state DC with low costimulation lead to regulatory responses against self-antigens, as was described in **chapter 1**. Observing the effects on phenotype and cytokine production, the question arose how histone deacetylase inhibition led to DC stuck in an immature state. Analyses on STAT phosphorylation showed that STAT signaling is interrupted in SAHA treated DC. Normally immature DC have a high level of phosphorylated STAT6 and a base level expression of phosphorylated STAT1. When a DC matures, the balance shifts to high levels of phosphorylated STAT1 and lower levels of STAT6. This switch in the life of a DC is crucial to mature and upregulate costimulation (268). SAHA interferes with this process by blocking the upregulation of STAT1 phosphorylation, which was independent of STAT6. In our experiments, we clearly demonstrate that DC were prone to mature in response to LPS as evidenced by the downregulation of STAT6 phosphorylation. Still no according rise in STAT1 phosphorylation was detected, indicating that SAHA generated DC are stuck in an immature state. Although STAT signaling can explain the observed effects, it is rather a combination of pathways that lead to the unresponsiveness and inactivated state of the investigated DC. Acetylation of several proteins influences protein function, signaling, stability and

protein/protein-DNA interactions (255). In this way, cellular biology is heavily affected by the use of HDAC inhibitors and is not limited to STAT signaling. Indeed, other molecules were affected by SAHA, more specifically TLR and MHC-II expression. A reduction in TLR2 can explain the insensitivity to LPS. It is hypothesized that TLR2 downregulation leads to a decreased signaling, thereby increasing the threshold that is needed to induce maturation in DC. The downregulation of TLR on SAHA generated DC is important in a successful therapy, when the DC are administered *in vivo*. The administered DC must retain their regulatory properties and not regain immune stimulatory functions in response to an inflammatory environment. With regard to the immune regulatory character, we found that SAHA generated DC are functionally hindered in the uptake and presentation of antigens and cytokine production. The reduced cytokine production can be the result of the block in STAT1 signaling and subsequent maturation, but may also be mediated through inhibition of NF-κB signaling. SAHA hyperacetylates the RelA subunit which blocks the translocation of NF-κB to the nucleus (277). Moreover lower nuclear levels of the RelB subunit are found when histone deacetylase inhibitors are used (315). Besides cytokine production, the observed block in myelin uptake and lower levels of MHC-II molecules detected after SAHA treatment *in vitro*, can be beneficial for the DC therapy. DC would be limited in the uptake and presentation of other antigens once injected *in vivo*, probably due to a downregulation of endocytosis receptors. In this way, peptides artificially loaded in the lab on these DC have the upper hand and lead to a more controlled and specific response. However we have to keep in mind that sufficient MHC-II molecules are needed to guarantee an antigen specific response. Otherwise the DC would induce systemic regulation against unwanted peptides or would not be able to interfere specifically with the target antigen.
As already mentioned the sensitivity to *in vivo* stimuli can undermine the tolerogenic potential of the DC therapy and switch the DC back to stimulating immune reactions. Although SAHA showed promising results on DC *in vitro*, the inefficiency *in vivo* led to the conclusion that these DC were not stable. Indeed when we removed SAHA from the cell cultures, DC regained their stimulatory potential indicated by the upregulation of the costimulatory molecules CD80 and CD86. Which parameters make the use of histone deacetylase inhibitors complicated in generating a tDC therapy to treat inflammatory diseases? Firstly, DC remain the most developmentally uncharacterized cell type within the immune

system. Although several restricted bone marrow precursors are found, it is still unknown where the initial commitment to the DC lineage and the split from monocytes starts. Secondly, research into the field of factors determining the differentiation of DC have defined crucial transcription factors such as Ikaros and PU.1. For example Ikaros-null mice lack the presence of pDC and $CD4^{+}$ cDC and mice bearing the dominant-negative form have an absence of all DC subsets. Furthermore the identification of E2-2 and Batf3 improved our understanding in the functional differences between pDC and cDC (316). However, there is still no consensus in the precise role of each DC lineage in inflammation and tolerance. Besides the complexity of the DC differentiation process, their functional maturation is another critical feature that is strictly regulated by a multitude of genes. A review of Dalod et al. clearly shows the involvement of several genes in the maturation process. However, even in DC maturation, different genes can be activated or inactivated based on DC undergoing homeostatic maturation (leading to tolerance) or maturation induced by TLR. The involvement of different genes and DC subtypes makes DC maturation a heterogeneous process that ultimately leads to a distinct functional DC (317). Clearly the role of genes and epigenetic mechanisms needs to be elucidated to evolve a network of transcription factors important in the various DC subsets that subsequently can be used to manipulate DC in a clinical setting. Identification of new regulation pathways by using histone deacetylase inhibitors contributes to our knowledge, but studying the subtle differences in epigenetics and their regulation in several DC subtypes is essential to take full advantage in exploiting this as a therapy. Moreover comparing different modulators is difficult due to a non-consensus between applied methods in similar studies. Studies need to test the stability of their therapy with several maturation inducing stimuli which, until now, is not always incorporated in the standard procedures referring back to the differences in genes involved in the maturation process. This is of course a major goal in the future of a tolerogenic DC therapy but in this thesis we have taken the first steps in unraveling the effect of epigenetic treatment on cell function, more specifically in this case DC.

Future perspectives to reveal the full potential of DC therapy

While our DC-based therapy showed no effects *in vivo*, epigenetic treatment of DC shows promise. The chance of side effects is reduced in our cell-based approach compared to systemic drug treatments. Our research focused on the use

of cDC. However, other DC subtypes should be considered for modulation. The first subtype that may be an alternative source are pDC. pDC produce already high levels of IFN (IFN therapy is used in MS) and therefore starting with pDC gives us already live reservoirs of IFN. However, pDC are difficult to culture and optimized protocols of acquiring sufficient numbers of pDC are lacking in contrast to the optimized culture methods of cDC (263, 264). A more specific response can be engineered by loading the DC with various antigens. But first we need a working and stable tolerogenic DC. One point of concern in our experiments was the presence of endogenous DC in our animals. This could further complicate the experiments, because injected DC come in an environment already stimulated by the endogenous DC. Therefore future research with DC needs to be done in CD11c-DTR animals where we can delete the endogenous DC to establish the effects of only the DC vaccine in question. These animals are ideally suited for our purposes of investigating the effect of SAHA-generated DC, because Yogev et al. demonstrated that CD11c-DTR animals do develop EAE and that initiation and primary activation of pathogenic T cells are independent of DC (185). Although ultimately a functional DC therapy has to override the endogenous DC, the exact *in vivo* mechanism as well as injection route of SAHA-generated DC can best be studied in CD11c-DTR animals. Another option is to treat animals with a short and low dose of SAHA to tune the endogenous DC towards tolerogenic. A combined boost with the DC-therapy may than be sufficient to re-establish tolerance. If we label the exogenous acquired DC, we can study the effect and interactions of not only *in vivo* manipulated DC, but also the injected DC. Lastly, we only explored the effect of a HDAC inhibitor as a tool to make tolerogenic DC, but other epigenetic mechanisms such as miRNA-induced silencing and DNA methylations can be investigated in their capacity to induce tolerogenic DC. We can conclude that the field of epigenetics is an interesting playground for researchers to be explored in the context of a cell-based therapy.

5.3 Aim III: Targetting regulatory mechanisms in vivo by DNA methylation inhibition to counteract EAE

Instead of *in vitro* generating a DC-based therapy for the treatment of autoimmune disorders, another strategy is to directly target regulatory mechanisms *in vivo*. The histone acetylase inhibitor vorinostat, with the active component SAHA, ameliorates EAE (225). Moreover a study done by Zhang and

colleagues revealed that miR-155 is correlated with disease severity in MS patients. Overexpression of this miRNA induced a more severe EAE with higher levels of Th1 and Th17 cells, whereas the opposite was true when working with miR-155 knock-out animals (318). Another recent study demonstrated that overexpression of miR-20b leads to lower levels of Th17 cells and milder EAE (319). These latest studies provide evidence to the new concept that epigenetic treatments are interesting tools to treat autoimmune disorders. However studies that directly intervene in DNA-methylations *in vivo* are still lacking. Therefore, in **chapter 4** we investigated if DNA methylation inhibition is a feasible therapeutic option for MS.

Are DNA methylation inhibitors a feasible option in multiple sclerosis?

To inhibit DNA methylations we chose to use decitabine (DAC) in our EAE model. DAC is an effective methylation inhibitor which induces hypomethylation by acting as a deoxyribonucleoside analogue. Hypomethylating agents do not target cells for immediate death, but cells are still able to undergo proliferation. In this way only active proliferating cells are affected leading to less side effects. Moreover genes that were previously methylation-silenced are reactivated. DAC has been widely studied in cancer research and is approved for the treatment of myelodysplasic syndrome (MDS) (217-221). It has been shown to improve outcomes of patients with MDS (217-219). Hypomethylation in this case is associated with reactivation of multiple genes, including tumor-suppressor genes and induction of cell death. Moreover clinical trials have confirmed that low-doses are associated with less toxicity (myelosuppression) and a greater response (222, 223) which makes DAC an interesting drug to explore in other settings, more specifically modulation of immune function. Studies in mouse models of asthma and diabetes showed therapeutic effects of DNA methylation inhibitors which was associated with an increase in regulatory T cells. In these studies the increase in regulatory T cells by the DNA methylation inhibitors was due to hypomethylation in the TSDR region of Foxp3 (226, 286). Based on these observations, we hypothesize that DAC may be of therapeutic value in MS by restoring regulatory T cell function. To test our hypothesis we intraperitoneally treated EAE mice on a daily base with 0.15 mg/kg DAC and evaluated disease severity and CNS immune infiltration as shown in detail in **chapter 4**. DAC-treated mice do not develop any

symptoms characteristic of EAE and this resistance to develop EAE was associated with reduced proliferation of spleen cells in response to MOG stimulus. Furthermore we observed a block of infiltration of macrophages and T cells into the spinal cord of treated mice. When treatment was stopped mice stayed clear of any symptoms, suggesting an induction of tolerance. Concomitant analyses on T cell compartments in primary and secondary lymphoid organs revealed that the number of T cells in the thymus was significantly reduced together with a rise in the percentage of Foxp3$^+$ T cells and reduced percentage of IL-17 producing cells within the CD4$^+$ T cells. This suggests a role for regulatory T cells in the observed effect. This effect on thymic output can be translated to reduced populations of effector T cells and higher levels of unaffected regulatory T cells in the periphery and hence the observed clinical effect. Given the important role of the thymus in the development of T cells, it can be speculated that a shrinkage of the thymus leads to a reduced thymic output of T cells in comparison with DMSO-treated EAE mice and healthy control mice. DAC may also induce regulatory T cells with a higher immunosuppressive capacity (226). In humans, DAC regulates Foxp3 expression in T cells by DNA methylation leading to functional and stable Treg (213). On the other hand, a recent human study with DAC done by Kehrmann et al., demonstrated that DAC-induced Tregs do not suppress the proliferation of responder cells. Furthermore, they did not find any relevant hypomethylations within the TSDR or expression of Treg specific genes (288). Because of this controversy, a follow-up study needs to investigate whether DAC leads to a stable induction of the regulatory phenotype and which precise pathways are involved in the observed effect. In this regard, analyses of the hypomethylation status of the TSDR region in untreated versus DAC treated EAE mice will be invaluable. Moreover co-culture experiments between MOG-reactive T cells and DAC treated spleen cells may provide the missing link between the observed increase in Foxp3 expression and the clinical effect and exclude other mechanisms involved.
Although future studies must elucidate the exact mechanism behind the effect of DAC, our study clearly proved that DAC can be explored as a possible new therapeutic approach to treat (auto)immune diseases.

5.4 General conclusion

Collectively, our results demonstrate the importance of epigenetic treatment as a therapeutic tool to restore tolerance in an autoimmune setting. First, we

elaborated further on the knowledge of DC and their role in the pathogenesis of MS. We clearly show that MS-associated genetic risk factors have an impact on the immune compartment in specific patients. Future association studies between risk factors and their impact on different cell types may make it easier to predict patient outcome and treatment responses. Second, we proved that epigenetic treatment is a viable and promising strategy not only for cell-based therapies but also to directly target regulatory mechanisms *in vivo*. In this regard, we provide evidence that inhibition of histone deacetylase and DNA methylation have an impact on the immune system skewing it towards tolerance and immune regulation. Our experiments, especially the use of DAC, show promise for patients suffering of an autoimmune or other inflammatory disease. Moreover, combination treatments that target several epigenetic mechanisms may further combat complicated diseases at several fronts, like MS. It is known that epigenetic mechanisms such as histone deacetylases and DNA methylations collaborate in silencing and controlling gene expression and therefore it warrants further research. A review by Suarez-Alvarez clearly describes how DNA methylation patters are modified during hematopoietic differentiation. Changes that occur in the DNA methylation profile have a crucial impact on the differentiation of hematopoietic cells towards myeloid and lymphoid lineages. This gives DNA methylations a crucial role in the establishment of specific phenotypes and functions of terminally differentiated cells (320). A limitation of our study is that we only examined the effect of DAC in the $CD4^+$ T cell compartment. It is likely that DAC also affects other immune cells such as $CD8^+$ T cells, B cells, macrophages and DC. Indeed, literature shows that DNA methylation and histone modifications at the transcription factor loci, T-bet, IFN_γ and IL-2 are essential for promoting the effector functions of $CD8^+$ T cells. Also, B cell differentiation and function is guided by DNA methylations. For example, the expression of B cell specific factors like Pax5 and rearrangements of the B-cell receptor are controlled by methylation at the DNA level. In addition, myeloid cells such as macrophages and DC are under epigenetic regulation. Histone modifications are important in M1 and M2 polarization of macrophages. So far, DNA methylations are not analysed for their impact in the macrophage department. It can be speculated that DNA methylations are involved in the expression of several cytokines important in macrophage polarization. DC differentiation out of monocytes is accompanied with a loss in DNA methylation on the level of DC-SIGN. DC-SIGN is

critical for DC trafficking and interactions with T cells (320). Further investigation of DAC and other DNA (de)methylation agents on different cell types are needed to understand the full activity of these drugs in modulating/polarizing the immune system under pathological and non-pathological conditions. Last, as is made clear in the previous chapters, epigenetics play an important role in the etiology of MS as multiple risk factors involve epigenetic modulations/alterations. Clinical severity and progression might be affected by epigenetic factors and explain certain features such as for example higher prevalence in women, reactivity against myelin, sun light exposure and viral infections. Future research aimed to investigate MS as an epigenetic disease can offer new tools in the diagnosis and treatment of MS.

6

Nederlandse samenvatting

6 Nederlandse samenvatting

MS is een complexe ziekte met veel bijdragende factoren. Hoewel de etiologie achter de ziekte nog niet gekend is, is het duidelijk dat het immuunsysteem ontregeld is. Geactiveerde autoreactieve T-cellen infiltreren het centraal zenuwstelsel in deze auto-immuunziekte en starten een ontstekingsreactie. Deze T-cellen zijn in staat om de centrale tolerantie te doorbreken die normaal ontstaat in de thymus. Hoewel er autoreactieve T-cellen aanwezig zijn bij gezonde personen, leiden die niet automatisch tot de ontwikkeling van auto-immuniteit. Dit suggereert de aanwezigheid van mechanismen die de activatie en expansie van autoreactieve T-cellen tegengaan. Voorgaande studies hebben aangetoond dat regulatoire T-cellen hierin een belangrijke rol vervullen. Bijvoorbeeld, $CD4^{+}CD25^{+}$ Tregs zijn minder functioneel in MS-patiënten. Tot op heden is nog niet bekend wat de oorzaak is van de verminderde aanwezigheid en functie van deze natuurlijke $CD4^{+}CD25^{+}$ Tregs bij MS-patiënten. De onlangs geïdentificeerde susceptibiliteitsgenen voor MS wijzen op een mogelijke genetische invloed in de waargenomen Treg dysfunctie. Een andere mogelijke reden is de uitputting van Tregs door de continue chronische ontsteking. Recent werd aangetoond dat dendritische cellen tevens een voorname rol spelen bij de homeostase van Tregs. DC behoren tot het aangeboren immuunsysteem en zijn algemeen gekend als de professionele antigeen-presenterende cellen. Deze cellen kunnen zowel immuniteit als tolerantie beïnvloeden. DC zijn niet alleen de belangrijkste induceerders van primaire T-cel-responsen, ze bepalen verder ook het profiel van deze reacties, afhankelijk van de co-stimulatie die zij verlenen. De groep van Darasse observeerde dat een verlies van deze DC in muizen leidt tot een vermindering van het aantal Tregs. Dit DC-afhankelijk verlies van Tregs ging gepaard met een toename van het aantal auto-agressieve T-cellen. Tevens nam men een verhoging waar van pro-inflammatoire cytokines. Vanwege hun unieke vermogen om immuunreacties te vormen, gaat men ervan uit dat DC een centrale rol spelen bij de immunopathogenese van verschillende auto-immuunziekten, waaronder MS.

Een tweede factor die een invloed heeft op immuunregulatie is epigenetische modificaties. Zo toonden studies aan dat DNA-methylatie en histon modificaties de expressie van genen geasscocieerd met het immuunsysteem regelen. Op deze

manier modificeren zij de ontwikkeling van aangeboren en verworven immuunreacties.

In dit proefschrift wordt dieper ingegaan op de **specifieke rol van DC in MS**. Hierbij wordt bepaald of circulerende types van DC veranderingen ondergaan in hun fenotypisch, functioneel en migratoire profiel. Dit werd onderzocht in een uitgebreide cohort van MS-patiënten tijdens verschillende stadia van de ziekte. Anderzijds **evalueerden we of targetting van DC of epigenetische modulatie het immuunevenwicht kan herstellen in een diermodel voor MS**, namelijk experimentele auto-immune encefalomyelitis. In de volgende paragrafen worden de belangrijkste resultaten samengevat.

Zijn er verschillen tussen het fenotypisch, functioneel en migratoir patroon van DC geïsoleerd uit MS en gezonde personen?

Algemeen wordt aanvaard dat MS zich ontwikkelt in genetisch vatbare personen als gevolg van een doorbreking van de tolerantie voor lichaamseigen eiwitten. Deze immuun-ontregeling in MS is uitgebreid gedocumenteerd op het niveau van het verworven immuunsysteem. Recente studies suggereren dat DC hierin ook een belangrijke rol spelen. In **hoofdstuk 2** zijn we dieper ingegaan op voorgaande studies en hebben we het fenotypisch, functioneel en migratoir profiel van circulerende DC in een uitgebreide cohort van MS-patiënten bepaald. Hierbij observeerden we een significante vermindering van circulerende pDC in patïenten met chronische progressieve MS in vergelijking met relapsing-remitting MS en gezonde personen. Verder werden er geen verschillen in cDC-frequentie gevonden tussen de verschillende onderzochte groepen. DC afkomstig van MS-patiënten vertoonden tevens een verhoogde IL-12p70 productie na TLR-ligatie en een verhoogde expressie van de migratiemoleculen CCR5 en CCR7. Dit vertaalde zich in een verhoogde *in vitro* chemotaxis naar de respectievelijke liganden van CCR5 en CCR7. Bovendien onderzochten we of het DC-compartiment werd beïnvloed door MS-gerelateerde genetische risico-allelen in specifieke immunologische genen zoals HLA-DRB1*1501 en IL-7Rα. HLA-DRB1*1501^{+} MS-patiënten en patiënten die niet drager zijn van het beschermende haplotype 2 van de IL-7Rα hebben verlaagde aantallen van respectievelijk cDC en pDC in hun bloed.

Onze waarnemingen wijzen op het belang van het bestuderen van verschillende DC-populaties in de context van MS. Elke subpopulatie heeft zijn eigen taken die kunnen worden verstoord en leiden tot de waargenomen individuele variaties in

ziekteverloop of symptomen tussen patiënten. Bovendien kunnen genetische risicofactoren en geassocieerde moleculen verder bijdragen tot de verhoogde heterogeniteit tussen MS-patiënten, resulterende in de complexiteit van deze ziekte. Als gevolg hiervan kunnen sommige patiënten beter profiteren van bepaalde behandelingen dan anderen. Daarom is het belangrijk dat verder onderzoek gebeurt met betrekking tot de verschillende immuun componenten en geassocieerde genetische factoren. Op deze manier krijgt men een gedetailleerde weergave van elke MS subpopulatie en kan men hierop de prognose en behandeling verder finetunen.

Kan men tolerantie-inducerende DC maken met de histone deacetylase inhibitor SAHA om immuuntolerantie te herstellen in EAE?

In **hoofdstuk 3** evauleerden we of de behandeling met SAHA leidde tot stabiele tolerante DC om op deze manier auto-immune reacties tegen te gaan in het diermodel voor MS, namelijk EAE. We onderzochten of we het tolerantie-inducerend vermogen van DC konden verhogen door deze cellen continu te behandelen met SAHA in vergelijking met de standaard 24u behandeling van reeds terminaal gedifferentieerde DC. In dit hoofdstuk hebben we aangetoond dat *in vitro* behandeling met SAHA de vorming van nieuwe CD11c^{+} DC uit muis beenmerg verminderde. Nieuwe gegenereerde DC met een 10d behandeling met SAHA bezaten meer eigenschappen van tolerantie-inducerende DC dan de standaardbehandeling. Deze DC hadden een afname in endocytose en antigeen-presenteerende capaciteit. Verder waren ze niet in staat om co-stimulatie moleculen te verhogen na stimulatie met LPS. Deze SAHA-gegenereerde DC hadden tevens een verlaagde productie van pro-inflammatoire cytokines en moleculen betrokken bij apoptose-inductie, migratie en TLR signalering. Toen we op zoek gingen naar het onderliggend mechanisme, observeerden we een verminderde STAT1 fosforylatie terwijl de effecten op DC onafhankelijk waren van STAT6 activatie. Hoewel deze *in vitro* resultaten veelbelovend waren, waren de SAHA-gegenereerde DC niet in staat om de ontwikkeling van auto-immune reacties tegen te gaan in EAE. Verdere *in vitro* experimenten toonden aan dat het tolerantie-inducerend fenotype van SAHA-gegenereerde DC omkeerbaar was.
Ter conclusie concludeerden we dat alhoewel SAHA een krachtige stimulus is van tolerogene karakteristieken in DC tijdens hun differentiatieproces, ze niet in staat

zijn om *in vivo* auto-immune reacties te stoppen. Daarom impliceren onze resultaten dat voorzichtigheid moet worden geboden bij de ontwikkeling van DC-gebaseerde behandelingen die tolerantie willen bewerkstellingen in de context van auto-immuunziekten.

Kan men door het *targetten* van epigenetische mechanismen auto-immune reacties tegengaan?

In plaats van het *in vitro* genereren van DC-gebaseerde behandelingen als therapie voor auto-immuunziekten, kan men als alternatieve strategie rechtstreeks *in vivo* gaan ingrijpen in regulerende mechanismen. In **hoofdstuk 4** hebben we onderzocht of het inhiberen van DNA-methylaties een haalbare therapeutische optie is voor de behandeling van MS. Om DNA-methylaties te remmen hebben we gekozen om gebruik te maken van decitabine (DAC). DAC is een effectieve methylatie remmer die hypomethylatie induceert door als een deoxyribonucleoside analoog te werken. Om onze hypothese te bevestigen werden EAE-muizen dagelijks behandeld met DAC en evalueerden we de ernst van de ziekte. Zoals in detail beschreven in **hoofdstuk 4** vertoonden DAC-behandelde muizen geen enkel symptoom van EAE dit vertaalde zich verder in verminderde proliferatieresponsen van miltcellen in respons op een MOG stimulus. Daarnaast namen we een blok waar in de infiltratie van macrofagen en T-cellen in het ruggenmerg van behandelde dieren. Ook wanneer de behandeling werd gestopt, bleven deze muizen symptoomvrij. Dit suggereert een inductie van tolerantie tegen MOG. Uit gelijktijdige analyses op T-cel compartimenten in primaire en secundaire lymfoïde organen is gebleken dat het aantal T-cellen in de thymus significant werd gereduceerd parallel met een stijging in het aantal Foxp3$^+$ T-cellen en een daling in het aantal IL-17 producerende T-cellen. Dit effect op de thymus kan vertaald worden naar een verminderde populatie van effector T-cellen en verhoogde aantallen van regulatoire T-cellen in de periferie met als resultaat het geobserveerde effect in DAC-behandelde muizen. Gezien de belangrijke rol van de thymus in de ontwikkeling van T-cellen kunnen we speculeren dat een krimping van de thymus leidt tot een verminderde productie van T-cellen in vergelijking met DMSO-behandelde EAE muizen en gezonde controlemuizen. Verder kan DAC ook regulerende T-cellen induceren met een hogere immunosuppressieve capaciteit. Aangezien het onderliggende mechanisme niet geheel duidelijk is, zal een follow-up studie moeten onderzoeken of DAC leidt tot een stabiele inductie van het

regulerende fenotype. In dit verband, kan men de hypomethylatie status analyzeren van de TSDR regio in onbehandelde versus DAC-behandeld EAE muizen. Bovendien kunnen co-cultuur experimenten tussen MOG-reactieve T-cellen en DAC-behandeld milt cellen leiden tot het onthullen van de ontbrekende schakel tussen de waargenomen stijging van de Foxp3 expressie en het klinische effect.

Algemene conclusie

Collectief tonen onze resultaten het belang van epigenetische modulatie aan als een therapeutisch middel om tolerantie te herstellen in een auto-immune setting. Eerst hebben we onze kennis van DC en hun rol in de pathogenese van MS verder uitgebreid. We hebben duidelijk bewezen dat MS-gerelateerde genetische risicofactoren van invloed zijn op het immuuncompartiment in specifieke patiënten. Toekomstige associatiestudies tussen risicofactoren en hun impact op verschillende celtypes kan het ons gemakkelijker maken om de patiënt uitkomst en de reacties op verschillende behandelingen te voorspellen. Ten tweede hebben we bewezen dat epigenetische modulatie een veelbelovende strategie is, niet alleen voor cel-gebaseerde therapieën, maar ook om regulerende mechanismen rechtstreeks *in vivo* te moduleren. In dit verband gaven wij met verschillende experimenten weer dat de remming van histon deacetylases en DNA-methylaties een impact hebben op het immuunsysteem en dit systeem eerder verschoof naar tolerantie en immuunregulatie. Onze experimenten, vooral het gebruik van DAC, zijn veelbelovend voor patiënten met een auto-immune of andere inflammatoire aandoening. Bovendien is een combinatie van behandelingen die zich op verschillende epigenetische mechanismen richt een goede strategie om complexe ziekten, zoals MS, te bestrijden op verschillende fronten. Het is bekend dat epigenetische mechanismen zoals histon deacetylases en DNA-methylaties samenwerken om de genexpressie te controleren. Ten slotte, kan epigenetica een belangrijke rol spelen in de etiologie van MS omdat meerdere risicofactoren epigenetische modulaties/wijzigingen veroorzaken. Het is geweten dat de klinische ernst en progressie kan worden beïnvloed door epigenetische factoren en deze factoren kunnen bepaalde kenmerken verklaren, zoals bijvoorbeeld de hogere prevalentie bij vrouwen, reactiviteit tegen myeline, blootstelling aan zonlicht en invloed van virale infecties. Toekomstig onderzoek gericht op MS te onderzoeken als een epigenetische ziekte kunnen nieuwe hulpmiddelen in de diagnose en behandeling van MS aan het licht brengen.

Reference list

1. Confavreux C, Vukusic S, Moreau T, Adeleine P. Relapses and progression of disability in multiple sclerosis. The New England journal of medicine. 2000;343(20):1430-8.
2. Poser CM, Brinar VV. The nature of multiple sclerosis. Clinical neurology and neurosurgery. 2004;106(3):159-71.
3. Hellings N, Raus J, Stinissen P. Insights into the immunopathogenesis of multiple sclerosis. Immunologic research. 2002;25(1):27-51.
4. Milo R, Kahana E. Multiple sclerosis: geoepidemiology, genetics and the environment. Autoimmunity reviews. 2010;9(5):A387-94.
5. Charcot J. Histologie de la sclérose en plaque: Gazette des Hôpitaux; 1868. 41:[554-66].
6. Karussis D. The diagnosis of multiple sclerosis and the various related demyelinating syndromes: A critical review. Journal of autoimmunity. 2014;48-49C:134-42.
7. Katz Sand IB, Lublin FD. Diagnosis and differential diagnosis of multiple sclerosis. Continuum. 2013;19(4 Multiple Sclerosis):922-43.
8. Milo R, Miller A. Revised diagnostic criteria of multiple sclerosis. Autoimmunity reviews. 2014;13(4-5):518-24.
9. Schumacker GA, Beebe G, Kibler RF, Kurland LT, Kurtzke JF, McDowell F, et al. Problems of Experimental Trials of Therapy in Multiple Sclerosis: Report by the Panel on the Evaluation of Experimental Trials of Therapy in Multiple Sclerosis. Annals of the New York Academy of Sciences. 1965;122:552-68.
10. Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, et al. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. Annals of neurology. 1983;13(3):227-31.
11. McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Annals of neurology. 2001;50(1):121-7.
12. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Annals of neurology. 2011;69(2):292-302.
13. Miller D, Barkhof F, Montalban X, Thompson A, Filippi M. Clinically isolated syndromes suggestive of multiple sclerosis, part I: natural history, pathogenesis, diagnosis, and prognosis. Lancet neurology. 2005;4(5):281-8.
14. Compston A, Coles A. Multiple sclerosis. Lancet. 2008;372(9648):1502-17.
15. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L. Axonal transection in the lesions of multiple sclerosis. The New England journal of medicine. 1998;338(5):278-85.

16. Bar-Or A, Oliveira EM, Anderson DE, Hafler DA. Molecular pathogenesis of multiple sclerosis. Journal of neuroimmunology. 1999;100(1-2):252-9.
17. Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. The New England journal of medicine. 2000;343(13):938-52.
18. Sadovnick AD, Baird PA. The familial nature of multiple sclerosis: age-corrected empiric recurrence risks for children and siblings of patients. Neurology. 1988;38(6):990-1.
19. Ebers GC, Bulman DE, Sadovnick AD, Paty DW, Warren S, Hader W, et al. A population-based study of multiple sclerosis in twins. The New England journal of medicine. 1986;315(26):1638-42.
20. Hafler DA, Slavik JM, Anderson DE, O'Connor KC, De Jager P, Baecher-Allan C. Multiple sclerosis. Immunological reviews. 2005;204:208-31.
21. Hewagama A, Richardson B. The genetics and epigenetics of autoimmune diseases. Journal of autoimmunity. 2009;33(1):3-11.
22. Walsh EC, Guschwan-McMahon S, Daly MJ, Hafler DA, Rioux JD. Genetic analysis of multiple sclerosis. Journal of autoimmunity. 2003;21(2):111-6.
23. Schmidt H, Williamson D, Ashley-Koch A. HLA-DR15 haplotype and multiple sclerosis: a HuGE review. American journal of epidemiology. 2007;165(10):1097-109.
24. International Multiple Sclerosis Genetics C, Wellcome Trust Case Control C, Sawcer S, Hellenthal G, Pirinen M, Spencer CC, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature. 2011;476(7359):214-9.
25. Broux B, Hellings N, Venken K, Rummens JL, Hensen K, Van Wijmeersch B, et al. Haplotype 4 of the multiple sclerosis-associated interleukin-7 receptor alpha gene influences the frequency of recent thymic emigrants. Genes and immunity. 2010;11(4):326-33.
26. Cree BA, Rioux JD, McCauley JL, Gourraud PA, Goyette P, McElroy J, et al. A major histocompatibility Class I locus contributes to multiple sclerosis susceptibility independently from HLA-DRB1*15:01. PloS one. 2010;5(6):e11296.
27. De Jager PL, Baecher-Allan C, Maier LM, Arthur AT, Ottoboni L, Barcellos L, et al. The role of the CD58 locus in multiple sclerosis. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(13):5264-9.
28. International Multiple Sclerosis Genetics C, Hafler DA, Compston A, Sawcer S, Lander ES, Daly MJ, et al. Risk alleles for multiple sclerosis identified by a genomewide study. The New England journal of medicine. 2007;357(9):851-62.
29. Patsopoulos NA, Bayer Pharma MSGWG, Steering Committees of Studies Evaluating I-b, a CCRA, Consortium AN, GeneMsa, et al. Genome-wide meta-analysis identifies novel multiple sclerosis susceptibility loci. Annals of neurology. 2011;70(6):897-912.

30. Dyment DA, Ebers GC, Sadovnick AD. Genetics of multiple sclerosis. Lancet neurology. 2004;3(2):104-10.
31. Nischwitz S, Cepok S, Kroner A, Wolf C, Knop M, Muller-Sarnowski F, et al. More CLEC16A gene variants associated with multiple sclerosis. Acta neurologica Scandinavica. 2011;123(6):400-6.
32. De Jager PL, Jia X, Wang J, de Bakker PI, Ottoboni L, Aggarwal NT, et al. Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci. Nature genetics. 2009;41(7):776-82.
33. International Multiple Sclerosis Genetics C, Beecham AH, Patsopoulos NA, Xifara DK, Davis MF, Kemppinen A, et al. Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis. Nature genetics. 2013;45(11):1353-60.
34. Gale CR, Martyn CN. Migrant studies in multiple sclerosis. Progress in neurobiology. 1995;47(4-5):425-48.
35. Chan WW. Eskimos and multiple sclerosis. Lancet. 1977;1(8026):1370.
36. Kurtzke JF. Epidemiology and etiology of multiple sclerosis. Physical medicine and rehabilitation clinics of North America. 2005;16(2):327-49.
37. Koch MW, Metz LM, Agrawal SM, Yong VW. Environmental factors and their regulation of immunity in multiple sclerosis. Journal of the neurological sciences. 2013;324(1-2):10-6.
38. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part II: Noninfectious factors. Annals of neurology. 2007;61(6):504-13.
39. Goldacre MJ, Seagroatt V, Yeates D, Acheson ED. Skin cancer in people with multiple sclerosis: a record linkage study. Journal of epidemiology and community health. 2004;58(2):142-4.
40. Su Z, Narla SN, Zhu Y. 25-Hydroxyvitamin D: Analysis and clinical application. Clinica chimica acta; international journal of clinical chemistry. 2014;433C:200-5.
41. Ascherio A, Munger KL, Simon KC. Vitamin D and multiple sclerosis. Lancet neurology. 2010;9(6):599-612.
42. Hayes CE. Vitamin D: a natural inhibitor of multiple sclerosis. The Proceedings of the Nutrition Society. 2000;59(4):531-5.
43. van Etten E, Mathieu C. Immunoregulation by 1,25-dihydroxyvitamin D3: basic concepts. The Journal of steroid biochemistry and molecular biology. 2005;97(1-2):93-101.
44. Gilden DH. Infectious causes of multiple sclerosis. Lancet neurology. 2005;4(3):195-202.
45. Sotelo J, Ordonez G, Pineda B. Varicella-zoster virus at relapses of multiple sclerosis. Journal of neurology. 2007;254(4):493-500.
46. Tang YW, Sriram S, Li H, Yao SY, Meng S, Mitchell WM, et al. Qualitative and quantitative detection of Chlamydophila pneumoniae DNA in cerebrospinal fluid from multiple sclerosis patients and controls. PloS one. 2009;4(4):e5200.

47. Ascherio A, Munger KL. Epstein-barr virus infection and multiple sclerosis: a review. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology. 2010;5(3):271-7.
48. Salvetti M, Giovannoni G, Aloisi F. Epstein-Barr virus and multiple sclerosis. Current opinion in neurology. 2009;22(3):201-6.
49. Ghadirian P, Jain M, Ducic S, Shatenstein B, Morisset R. Nutritional factors in the aetiology of multiple sclerosis: a case-control study in Montreal, Canada. International journal of epidemiology. 1998;27(5):845-52.
50. Hernan MA, Olek MJ, Ascherio A. Cigarette smoking and incidence of multiple sclerosis. American journal of epidemiology. 2001;154(1):69-74.
51. Riccio P. The molecular basis of nutritional intervention in multiple sclerosis: a narrative review. Complementary therapies in medicine. 2011;19(4):228-37.
52. Muldoon LL, Alvarez JI, Begley DJ, Boado RJ, Del Zoppo GJ, Doolittle ND, et al. Immunologic privilege in the central nervous system and the blood-brain barrier. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 2013;33(1):13-21.
53. Owens T, Bechmann I, Engelhardt B. Perivascular spaces and the two steps to neuroinflammation. Journal of neuropathology and experimental neurology. 2008;67(12):1113-21.
54. Ransohoff RM, Kivisakk P, Kidd G. Three or more routes for leukocyte migration into the central nervous system. Nature reviews Immunology. 2003;3(7):569-81.
55. Greter M, Heppner FL, Lemos MP, Odermatt BM, Goebels N, Laufer T, et al. Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. Nature medicine. 2005;11(3):328-34.
56. Wilson EH, Weninger W, Hunter CA. Trafficking of immune cells in the central nervous system. The Journal of clinical investigation. 2010;120(5):1368-79.
57. Costantino CM, Baecher-Allan C, Hafler DA. Multiple sclerosis and regulatory T cells. Journal of clinical immunology. 2008;28(6):697-706.
58. Ota K, Matsui M, Milford EL, Mackin GA, Weiner HL, Hafler DA. T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. Nature. 1990;346(6280):183-7.
59. Hauser SL, Oksenberg JR. The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration. Neuron. 2006;52(1):61-76.
60. Platten M, Steinman L. Multiple sclerosis: trapped in deadly glue. Nature medicine. 2005;11(3):252-3.
61. Stys PK, Zamponi GW, van Minnen J, Geurts JJ. Will the real multiple sclerosis please stand up? Nature reviews Neuroscience. 2012;13(7):507-14.
62. Trapp BD, Nave KA. Multiple sclerosis: an immune or neurodegenerative disorder? Annual review of neuroscience. 2008;31:247-69.

63. Chitnis T. The role of CD4 T cells in the pathogenesis of multiple sclerosis. International review of neurobiology. 2007;79:43-72.
64. Broux B, Stinissen P, Hellings N. Which immune cells matter? The immunopathogenesis of multiple sclerosis. Critical reviews in immunology. 2013;33(4):283-306.
65. LaSalle JM, Ota K, Hafler DA. Presentation of autoantigen by human T cells. J Immunol. 1991;147(3):774-80.
66. Pelfrey CM, Rudick RA, Cotleur AC, Lee JC, Tary-Lehmann M, Lehmann PV. Quantification of self-recognition in multiple sclerosis by single-cell analysis of cytokine production. J Immunol. 2000;165(3):1641-51.
67. Zhang J, Markovic-Plese S, Lacet B, Raus J, Weiner HL, Hafler DA. Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. J Exp Med. 1994;179(3):973-84.
68. Merrill JE. Proinflammatory and antiinflammatory cytokines in multiple sclerosis and central nervous system acquired immunodeficiency syndrome. Journal of immunotherapy : official journal of the Society for Biological Therapy. 1992;12(3):167-70.
69. Wucherpfennig KW, Catz I, Hausmann S, Strominger JL, Steinman L, Warren KG. Recognition of the immunodominant myelin basic protein peptide by autoantibodies and HLA-DR2-restricted T cell clones from multiple sclerosis patients. Identity of key contact residues in the B-cell and T-cell epitopes. The Journal of clinical investigation. 1997;100(5):1114-22.
70. Wucherpfennig KW, Hafler DA, Strominger JL. Structure of human T-cell receptors specific for an immunodominant myelin basic protein peptide: positioning of T-cell receptors on HLA-DR2/peptide complexes. Proceedings of the National Academy of Sciences of the United States of America. 1995;92(19):8896-900.
71. Wucherpfennig KW, Sette A, Southwood S, Oseroff C, Matsui M, Strominger JL, et al. Structural requirements for binding of an immunodominant myelin basic protein peptide to DR2 isotypes and for its recognition by human T cell clones. J Exp Med. 1994;179(1):279-90.
72. Pettinelli CB, McFarlin DE. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1+ 2- T lymphocytes. J Immunol. 1981;127(4):1420-3.
73. Durelli L, Conti L, Clerico M, Boselli D, Contessa G, Ripellino P, et al. T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-beta. Annals of neurology. 2009;65(5):499-509.
74. Kebir H, Kreymborg K, Ifergan I, Dodelet-Devillers A, Cayrol R, Bernard M, et al. Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. Nature medicine. 2007;13(10):1173-5.

75. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med. 2005;201(2):233-40.
76. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annu Rev Immunol. 2009;27:485-517.
77. McFarland HF, Martin R. Multiple sclerosis: a complicated picture of autoimmunity. Nat Immunol. 2007;8(9):913-9.
78. Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature. 2003;421(6924):744-8.
79. Goverman J. Autoimmune T cell responses in the central nervous system. Nature reviews Immunology. 2009;9(6):393-407.
80. Reboldi A, Coisne C, Baumjohann D, Benvenuto F, Bottinelli D, Lira S, et al. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. Nat Immunol. 2009;10(5):514-23.
81. Lim HW, Hillsamer P, Banham AH, Kim CH. Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. J Immunol. 2005;175(7):4180-3.
82. Taams LS, van Amelsfort JM, Tiemessen MM, Jacobs KM, de Jong EC, Akbar AN, et al. Modulation of monocyte/macrophage function by human CD4+CD25+ regulatory T cells. Human immunology. 2005;66(3):222-30.
83. Haas J, Hug A, Viehover A, Fritzsching B, Falk CS, Filser A, et al. Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. European journal of immunology. 2005;35(11):3343-52.
84. Kumar M, Putzki N, Limmroth V, Remus R, Lindemann M, Knop D, et al. CD4+CD25+FoxP3+ T lymphocytes fail to suppress myelin basic protein-induced proliferation in patients with multiple sclerosis. Journal of neuroimmunology. 2006;180(1-2):178-84.
85. Venken K, Hellings N, Broekmans T, Hensen K, Rummens JL, Stinissen P. Natural naive CD4+CD25+CD127low regulatory T cell (Treg) development and function are disturbed in multiple sclerosis patients: recovery of memory Treg homeostasis during disease progression. J Immunol. 2008;180(9):6411-20.
86. Venken K, Hellings N, Hensen K, Rummens JL, Medaer R, D'Hooghe M B, et al. Secondary progressive in contrast to relapsing-remitting multiple sclerosis patients show a normal CD4+CD25+ regulatory T-cell function and FOXP3 expression. J Neurosci Res. 2006;83(8):1432-46.
87. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J Exp Med. 2004;199(7):971-9.

88. Jacobsen M, Cepok S, Quak E, Happel M, Gaber R, Ziegler A, et al. Oligoclonal expansion of memory CD8+ T cells in cerebrospinal fluid from multiple sclerosis patients. Brain. 2002;125(Pt 3):538-50.
89. Saxena A, Martin-Blondel G, Mars LT, Liblau RS. Role of CD8 T cell subsets in the pathogenesis of multiple sclerosis. FEBS letters. 2011;585(23):3758-63.
90. Zang YC, Li S, Rivera VM, Hong J, Robinson RR, Breitbach WT, et al. Increased CD8+ cytotoxic T cell responses to myelin basic protein in multiple sclerosis. J Immunol. 2004;172(8):5120-7.
91. Biddison WE, Cruikshank WW, Center DM, Pelfrey CM, Taub DD, Turner RV. CD8+ myelin peptide-specific T cells can chemoattract CD4+ myelin peptide-specific T cells: importance of IFN-inducible protein 10. J Immunol. 1998;160(1):444-8.
92. Disanto G, Morahan JM, Barnett MH, Giovannoni G, Ramagopalan SV. The evidence for a role of B cells in multiple sclerosis. Neurology. 2012;78(11):823-32.
93. Archelos JJ, Storch MK, Hartung HP. The role of B cells and autoantibodies in multiple sclerosis. Annals of neurology. 2000;47(6):694-706.
94. Genain CP, Cannella B, Hauser SL, Raine CS. Identification of autoantibodies associated with myelin damage in multiple sclerosis. Nature medicine. 1999;5(2):170-5.
95. Warren KG, Catz I. An extensive search for autoantibodies to myelin basic protein in cerebrospinal fluid of non-multiple-sclerosis patients: implications for the pathogenesis of multiple sclerosis. European neurology. 1999;42(2):95-104.
96. Correale J, de los Milagros Bassani Molinas M. Oligoclonal bands and antibody responses in multiple sclerosis. Journal of neurology. 2002;249(4):375-89.
97. Lucchinetti C, Bruck W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. Annals of neurology. 2000;47(6):707-17.
98. Prineas JW, Graham JS. Multiple sclerosis: capping of surface immunoglobulin G on macrophages engaged in myelin breakdown. Annals of neurology. 1981;10(2):149-58.
99. Antel J, Bar-Or A. Roles of immunoglobulins and B cells in multiple sclerosis: from pathogenesis to treatment. Journal of neuroimmunology. 2006;180(1-2):3-8.
100. Monson NL, Cravens PD, Frohman EM, Hawker K, Racke MK. Effect of rituximab on the peripheral blood and cerebrospinal fluid B cells in patients with primary progressive multiple sclerosis. Arch Neurol. 2005;62(2):258-64.
101. Stuve O, Cepok S, Elias B, Saleh A, Hartung HP, Hemmer B, et al. Clinical stabilization and effective B-lymphocyte depletion in the cerebrospinal fluid and peripheral blood of a patient with fulminant relapsing-remitting multiple sclerosis. Arch Neurol. 2005;62(10):1620-3.

102. Bruck W, Porada P, Poser S, Rieckmann P, Hanefeld F, Kretzschmar HA, et al. Monocyte/macrophage differentiation in early multiple sclerosis lesions. Annals of neurology. 1995;38(5):788-96.
103. van der Laan LJ, Ruuls SR, Weber KS, Lodder IJ, Dopp EA, Dijkstra CD. Macrophage phagocytosis of myelin in vitro determined by flow cytometry: phagocytosis is mediated by CR3 and induces production of tumor necrosis factor-alpha and nitric oxide. Journal of neuroimmunology. 1996;70(2):145-52.
104. Selmaj KW, Raine CS. Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. Annals of neurology. 1988;23(4):339-46.
105. Hendriks JJ, Teunissen CE, de Vries HE, Dijkstra CD. Macrophages and neurodegeneration. Brain research Brain research reviews. 2005;48(2):185-95.
106. Glabinski A, Tawsek NS, Bartosz G. Increased generation of superoxide radicals in the blood of MS patients. Acta neurologica Scandinavica. 1993;88(3):174-7.
107. Lu F, Selak M, O'Connor J, Croul S, Lorenzana C, Butunoi C, et al. Oxidative damage to mitochondrial DNA and activity of mitochondrial enzymes in chronic active lesions of multiple sclerosis. Journal of the neurological sciences. 2000;177(2):95-103.
108. Vladimirova O, Lu FM, Shawver L, Kalman B. The activation of protein kinase C induces higher production of reactive oxygen species by mononuclear cells in patients with multiple sclerosis than in controls. Inflammation research : official journal of the European Histamine Research Society [et al]. 1999;48(7):412-6.
109. Giovannoni G. Cerebrospinal fluid and serum nitric oxide metabolites in patients with multiple sclerosis. Multiple sclerosis. 1998;4(1):27-30.
110. Boven LA, Van Meurs M, Van Zwam M, Wierenga-Wolf A, Hintzen RQ, Boot RG, et al. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. Brain. 2006;129(Pt 2):517-26.
111. Oberheim NA, Goldman SA, Nedergaard M. Heterogeneity of astrocytic form and function. Methods in molecular biology. 2012;814:23-45.
112. Nair A, Frederick TJ, Miller SD. Astrocytes in multiple sclerosis: a product of their environment. Cellular and molecular life sciences : CMLS. 2008;65(17):2702-20.
113. Miljkovic D, Timotijevic G, Mostarica Stojkovic M. Astrocytes in the tempest of multiple sclerosis. FEBS letters. 2011;585(23):3781-8.
114. Verkhratsky A, Rodriguez JJ, Parpura V. Astroglia in neurological diseases. Future neurology. 2013;8(2):149-58.
115. McCarthy DP, Richards MH, Miller SD. Mouse models of multiple sclerosis: experimental autoimmune encephalomyelitis and Theiler's virus-induced demyelinating disease. Methods in molecular biology. 2012;900:381-401.

116. Goverman J, Woods A, Larson L, Weiner LP, Hood L, Zaller DM. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. Cell. 1993;72(4):551-60.
117. Paterson PY. Transfer of allergic encephalomyelitis in rats by means of lymph node cells. J Exp Med. 1960;111:119-36.
118. Raine CS, Mokhtarian F, McFarlin DE. Adoptively transferred chronic relapsing experimental autoimmune encephalomyelitis in the mouse. Neuropathologic analysis. Laboratory investigation; a journal of technical methods and pathology. 1984;51(5):534-46.
119. Batoulis H, Recks MS, Addicks K, Kuerten S. Experimental autoimmune encephalomyelitis--achievements and prospective advances. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica. 2011;119(12):819-30.
120. Rangachari M, Kuchroo VK. Using EAE to better understand principles of immune function and autoimmune pathology. Journal of autoimmunity. 2013;45:31-9.
121. Comi G. Disease-modifying treatments for progressive multiple sclerosis. Multiple sclerosis. 2013;19(11):1428-36.
122. Lopez-Diego RS, Weiner HL. Novel therapeutic strategies for multiple sclerosis--a multifaceted adversary. Nature reviews Drug discovery. 2008;7(11):909-25.
123. Minagar A. Current and future therapies for multiple sclerosis. Scientifica. 2013;2013:249101.
124. Wingerchuk DM, Carter JL. Multiple sclerosis: current and emerging disease-modifying therapies and treatment strategies. Mayo Clinic proceedings. 2014;89(2):225-40.
125. Minagar A, Alexander JS, Schwendimann RN, Kelley RE, Gonzalez-Toledo E, Jimenez JJ, et al. Combination therapy with interferon beta-1a and doxycycline in multiple sclerosis: an open-label trial. Arch Neurol. 2008;65(2):199-204.
126. Prat A, Al-Asmi A, Duquette P, Antel JP. Lymphocyte migration and multiple sclerosis: relation with disease course and therapy. Annals of neurology. 1999;46(2):253-6.
127. Huang YM, Stoyanova N, Jin YP, Teleshova N, Hussien Y, Xiao BG, et al. Altered phenotype and function of blood dendritic cells in multiple sclerosis are modulated by IFN-beta and IL-10. Clin Exp Immunol. 2001;124(2):306-14.
128. Ozenci V, Kouwenhoven M, Teleshova N, Pashenkov M, Fredrikson S, Link H. Multiple sclerosis: pro- and anti-inflammatory cytokines and metalloproteinases are affected differentially by treatment with IFN-beta. Journal of neuroimmunology. 2000;108(1-2):236-43.
129. Zang Y, Hong J, Robinson R, Li S, Rivera VM, Zhang JZ. Immune regulatory properties and interactions of copolymer-I and beta-interferon 1a in multiple sclerosis. Journal of neuroimmunology. 2003;137(1-2):144-53.

130. Aharoni R, Kayhan B, Eilam R, Sela M, Arnon R. Glatiramer acetate-specific T cells in the brain express T helper 2/3 cytokines and brain-derived neurotrophic factor in situ. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(24):14157-62.
131. Aharoni R, Teitelbaum D, Sela M, Arnon R. Copolymer 1 induces T cells of the T helper type 2 that crossreact with myelin basic protein and suppress experimental autoimmune encephalomyelitis. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(20):10821-6.
132. Duda PW, Schmied MC, Cook SL, Krieger JI, Hafler DA. Glatiramer acetate (Copaxone) induces degenerate, Th2-polarized immune responses in patients with multiple sclerosis. The Journal of clinical investigation. 2000;105(7):967-76.
133. Hong J, Li N, Zhang X, Zheng B, Zhang JZ. Induction of CD4+CD25+ regulatory T cells by copolymer-I through activation of transcription factor Foxp3. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(18):6449-54.
134. Tennakoon DK, Mehta RS, Ortega SB, Bhoj V, Racke MK, Karandikar NJ. Therapeutic induction of regulatory, cytotoxic CD8+ T cells in multiple sclerosis. J Immunol. 2006;176(11):7119-29.
135. Hartung HP, Gonsette R, Konig N, Kwiecinski H, Guseo A, Morrissey SP, et al. Mitoxantrone in progressive multiple sclerosis: a placebo-controlled, double-blind, randomised, multicentre trial. Lancet. 2002;360(9350):2018-25.
136. Marriott JJ, Miyasaki JM, Gronseth G, O'Connor PW, Therapeutics, Technology Assessment Subcommittee of the American Academy of N. Evidence Report: The efficacy and safety of mitoxantrone (Novantrone) in the treatment of multiple sclerosis: Report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. Neurology. 2010;74(18):1463-70.
137. Engelhardt B, Kappos L. Natalizumab: targeting alpha4-integrins in multiple sclerosis. Neuro-degenerative diseases. 2008;5(1):16-22.
138. Sorensen PS, Bertolotto A, Edan G, Giovannoni G, Gold R, Havrdova E, et al. Risk stratification for progressive multifocal leukoencephalopathy in patients treated with natalizumab. Multiple sclerosis. 2012;18(2):143-52.
139. Chiba K. FTY720, a new class of immunomodulator, inhibits lymphocyte egress from secondary lymphoid tissues and thymus by agonistic activity at sphingosine 1-phosphate receptors. Pharmacology & therapeutics. 2005;108(3):308-19.
140. Cohen JA, Chun J. Mechanisms of fingolimod's efficacy and adverse effects in multiple sclerosis. Annals of neurology. 2011;69(5):759-77.
141. Noda H, Takeuchi H, Mizuno T, Suzumura A. Fingolimod phosphate promotes the neuroprotective effects of microglia. Journal of neuroimmunology. 2013;256(1-2):13-8.

142. Cohen JA, Coles AJ, Arnold DL, Confavreux C, Fox EJ, Hartung HP, et al. Alemtuzumab versus interferon beta 1a as first-line treatment for patients with relapsing-remitting multiple sclerosis: a randomised controlled phase 3 trial. Lancet. 2012;380(9856):1819-28.
143. Coles AJ, Fox E, Vladic A, Gazda SK, Brinar V, Selmaj KW, et al. Alemtuzumab versus interferon beta-1a in early relapsing-remitting multiple sclerosis: post-hoc and subset analyses of clinical efficacy outcomes. Lancet neurology. 2011;10(4):338-48.
144. Jones JL, Anderson JM, Phuah CL, Fox EJ, Selmaj K, Margolin D, et al. Improvement in disability after alemtuzumab treatment of multiple sclerosis is associated with neuroprotective autoimmunity. Brain. 2010;133(Pt 8):2232-47.
145. Fox RJ, Miller DH, Phillips JT, Hutchinson M, Havrdova E, Kita M, et al. Placebo-controlled phase 3 study of oral BG-12 or glatiramer in multiple sclerosis. The New England journal of medicine. 2012;367(12):1087-97.
146. Gold R, Kappos L, Arnold DL, Bar-Or A, Giovannoni G, Selmaj K, et al. Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis. The New England journal of medicine. 2012;367(12):1098-107.
147. Havrdova E, Hutchinson M, Kurukulasuriya NC, Raghupathi K, Sweetser MT, Dawson KT, et al. Oral BG-12 (dimethyl fumarate) for relapsing-remitting multiple sclerosis: a review of DEFINE and CONFIRM. Evaluation of: Gold R, Kappos L, Arnold D, et al. Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis. N Engl J Med 2012;367:1098-107; and Fox RJ, Miller DH, Phillips JT, et al. Placebo-controlled phase 3 study of oral BG-12 or glatiramer in multiple sclerosis. N Engl J Med 2012;367:1087-97. Expert opinion on pharmacotherapy. 2013;14(15):2145-56.
148. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med. 1973;137(5):1142-62.
149. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392(6673):245-52.
150. Steinman RM. Dendritic cells: understanding immunogenicity. European journal of immunology. 2007;37 Suppl 1:S53-60.
151. Merad M, Manz MG. Dendritic cell homeostasis. Blood. 2009;113(15):3418-27.
152. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Annu Rev Immunol. 2000;18:767-811.
153. Serafini B, Rosicarelli B, Magliozzi R, Stigliano E, Capello E, Mancardi GL, et al. Dendritic cells in multiple sclerosis lesions: maturation stage, myelin uptake, and interaction with proliferating T cells. Journal of neuropathology and experimental neurology. 2006;65(2):124-41.
154. Collin M, Bigley V, Haniffa M, Hambleton S. Human dendritic cell deficiency: the missing ID? Nature reviews Immunology. 2011;11(9):575-83.

155. Doulatov S, Notta F, Eppert K, Nguyen LT, Ohashi PS, Dick JE. Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. Nat Immunol. 2010;11(7):585-93.
156. Boltjes A, van Wijk F. Human Dendritic Cell Functional Specialization in Steady-State and Inflammation. Front Immunol. 2014;5:131.
157. Chopin M, Allan RS, Belz GT. Transcriptional regulation of dendritic cell diversity. Front Immunol. 2012;3:26.
158. Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell. 2001;106(3):259-62.
159. Jongbloed SL, Kassianos AJ, McDonald KJ, Clark GJ, Ju X, Angel CE, et al. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J Exp Med. 2010;207(6):1247-60.
160. Poulin LF, Salio M, Griessinger E, Anjos-Afonso F, Craciun L, Chen JL, et al. Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. J Exp Med. 2010;207(6):1261-71.
161. Colonna M, Trinchieri G, Liu YJ. Plasmacytoid dendritic cells in immunity. Nat Immunol. 2004;5(12):1219-26.
162. Martin-Gayo E, Sierra-Filardi E, Corbi AL, Toribio ML. Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development. Blood. 2010;115(26):5366-75.
163. Mathan TS, Figdor CG, Buschow SI. Human plasmacytoid dendritic cells: from molecules to intercellular communication network. Front Immunol. 2013;4:372.
164. Gregorio J, Meller S, Conrad C, Di Nardo A, Homey B, Lauerma A, et al. Plasmacytoid dendritic cells sense skin injury and promote wound healing through type I interferons. J Exp Med. 2010;207(13):2921-30.
165. Serbina NV, Salazar-Mather TP, Biron CA, Kuziel WA, Pamer EG. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. Immunity. 2003;19(1):59-70.
166. Leon B, Lopez-Bravo M, Ardavin C. Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania. Immunity. 2007;26(4):519-31.
167. Chorro L, Sarde A, Li M, Woollard KJ, Chambon P, Malissen B, et al. Langerhans cell (LC) proliferation mediates neonatal development, homeostasis, and inflammation-associated expansion of the epidermal LC network. J Exp Med. 2009;206(13):3089-100.
168. Nagao K, Ginhoux F, Leitner WW, Motegi S, Bennett CL, Clausen BE, et al. Murine epidermal Langerhans cells and langerin-expressing dermal dendritic cells are unrelated and exhibit distinct functions. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(9):3312-7.

169. Shklovskaya E, O'Sullivan BJ, Ng LG, Roediger B, Thomas R, Weninger W, et al. Langerhans cells are precommitted to immune tolerance induction. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(44):18049-54.
170. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. Science. 2010;327(5966):656-61.
171. Allan RS, Waithman J, Bedoui S, Jones CM, Villadangos JA, Zhan Y, et al. Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. Immunity. 2006;25(1):153-62.
172. Nairn R, Helbert M. Immunology : for medical students. Edinburgh: Mosby; 2002. 14 , 326 p. p.
173. Banchereau J, Palucka AK. Dendritic cells as therapeutic vaccines against cancer. Nat Rev Immunol. 2005;5(4):296-306.
174. Cools N, Ponsaerts P, Van Tendeloo VF, Berneman ZN. Balancing between immunity and tolerance: an interplay between dendritic cells, regulatory T cells, and effector T cells. J Leukoc Biol. 2007;82(6):1365-74.
175. Tan JK, O'Neill HC. Maturation requirements for dendritic cells in T cell stimulation leading to tolerance versus immunity. J Leukoc Biol. 2005;78(2):319-24.
176. Karni A, Abraham M, Monsonego A, Cai G, Freeman GJ, Hafler D, et al. Innate immunity in multiple sclerosis: myeloid dendritic cells in secondary progressive multiple sclerosis are activated and drive a proinflammatory immune response. J Immunol. 2006;177(6):4196-202.
177. Pashenkov M, Teleshova N, Kouwenhoven M, Kostulas V, Huang YM, Soderstrom M, et al. Elevated expression of CCR5 by myeloid (CD11c+) blood dendritic cells in multiple sclerosis and acute optic neuritis. Clin Exp Immunol. 2002;127(3):519-26.
178. Stasiolek M, Bayas A, Kruse N, Wieczarkowiecz A, Toyka KV, Gold R, et al. Impaired maturation and altered regulatory function of plasmacytoid dendritic cells in multiple sclerosis. Brain. 2006;129(Pt 5):1293-305.
179. Gandhi R, Laroni A, Weiner HL. Role of the innate immune system in the pathogenesis of multiple sclerosis. Journal of neuroimmunology.221(1-2):7-14.
180. Nuyts AH, Lee WP, Bashir-Dar R, Berneman ZN, Cools N. Dendritic cells in multiple sclerosis: key players in the immunopathogenesis, key players for new cellular immunotherapies? Multiple sclerosis. 2013;19(8):995-1002.
181. Huang YM, Xiao BG, Ozenci V, Kouwenhoven M, Teleshova N, Fredrikson S, et al. Multiple sclerosis is associated with high levels of circulating dendritic cells secreting pro-inflammatory cytokines. Journal of neuroimmunology. 1999;99(1):82-90.

182. Bailey SL, Schreiner B, McMahon EJ, Miller SD. CNS myeloid DCs presenting endogenous myelin peptides 'preferentially' polarize CD4+ T(H)-17 cells in relapsing EAE. Nat Immunol. 2007;8(2):172-80.
183. Serafini B, Columba-Cabezas S, Di Rosa F, Aloisi F. Intracerebral recruitment and maturation of dendritic cells in the onset and progression of experimental autoimmune encephalomyelitis. The American journal of pathology. 2000;157(6):1991-2002.
184. Isaksson M, Lundgren BA, Ahlgren KM, Kampe O, Lobell A. Conditional DC depletion does not affect priming of encephalitogenic Th cells in EAE. European journal of immunology. 2012;42(10):2555-63.
185. Yogev N, Frommer F, Lukas D, Kautz-Neu K, Karram K, Ielo D, et al. Dendritic cells ameliorate autoimmunity in the CNS by controlling the homeostasis of PD-1 receptor(+) regulatory T cells. Immunity. 2012;37(2):264-75.
186. Becher B, Greter M. Acquitting an APC: DCs found "not guilty" after trial by ablation. European journal of immunology. 2012;42(10):2551-4.
187. Reinhard G, Marten A, Kiske SM, Feil F, Bieber T, Schmidt-Wolf IG. Generation of dendritic cell-based vaccines for cancer therapy. British journal of cancer. 2002;86(10):1529-33.
188. Comabella M, Montalban X, Munz C, Lunemann JD. Targeting dendritic cells to treat multiple sclerosis. Nature reviews Neurology. 2010;6(9):499-507.
189. Hackstein H, Thomson AW. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. Nature reviews Immunology. 2004;4(1):24-34.
190. Cools N, Van Tendeloo VF, Smits EL, Lenjou M, Nijs G, Van Bockstaele DR, et al. Immunosuppression induced by immature dendritic cells is mediated by TGF-beta/IL-10 double-positive CD4+ regulatory T cells. Journal of cellular and molecular medicine. 2008;12(2):690-700.
191. Nikolic T, Roep BO. Regulatory multitasking of tolerogenic dendritic cells - lessons taken from vitamin d3-treated tolerogenic dendritic cells. Front Immunol. 2013;4:113.
192. Unger WW, Laban S, Kleijwegt FS, van der Slik AR, Roep BO. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. European journal of immunology. 2009;39(11):3147-59.
193. Menges M, Rossner S, Voigtlander C, Schindler H, Kukutsch NA, Bogdan C, et al. Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. J Exp Med. 2002;195(1):15-21.
194. Terness P, Oelert T, Ehser S, Chuang JJ, Lahdou I, Kleist C, et al. Mitomycin C-treated dendritic cells inactivate autoreactive T cells: toward the development of a tolerogenic vaccine in autoimmune diseases. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(47):18442-7.

195. Yang JS, Xu LY, Huang YM, Van Der Meide PH, Link H, Xiao BG. Adherent dendritic cells expressing high levels of interleukin-10 and low levels of interleukin-12 induce antigen-specific tolerance to experimental autoimmune encephalomyelitis. Immunology. 2000;101(3):397-403.
196. Huang YM, Yang JS, Xu LY, Link H, Xiao BG. Autoantigen-pulsed dendritic cells induce tolerance to experimental allergic encephalomyelitis (EAE) in Lewis rats. Clin Exp Immunol. 2000;122(3):437-44.
197. Iruretagoyena MI, Sepulveda SE, Lezana JP, Hermoso M, Bronfman M, Gutierrez MA, et al. Inhibition of nuclear factor-kappa B enhances the capacity of immature dendritic cells to induce antigen-specific tolerance in experimental autoimmune encephalomyelitis. The Journal of pharmacology and experimental therapeutics. 2006;318(1):59-67.
198. Ilarregui JM, Croci DO, Bianco GA, Toscano MA, Salatino M, Vermeulen ME, et al. Tolerogenic signals delivered by dendritic cells to T cells through a galectin-1-driven immunoregulatory circuit involving interleukin 27 and interleukin 10. Nat Immunol. 2009;10(9):981-91.
199. Koch MW, Metz LM, Kovalchuk O. Epigenetic changes in patients with multiple sclerosis. Nature reviews Neurology. 2013;9(1):35-43.
200. Huynh JL, Casaccia P. Epigenetic mechanisms in multiple sclerosis: implications for pathogenesis and treatment. Lancet neurology. 2013;12(2):195-206.
201. Meda F, Folci M, Baccarelli A, Selmi C. The epigenetics of autoimmunity. Cellular & molecular immunology. 2011;8(3):226-36.
202. Javierre BM, Hernando H, Ballestar E. Environmental triggers and epigenetic deregulation in autoimmune disease. Discovery medicine. 2011;12(67):535-45.
203. Liggett TE, Melnikov AA, Marks JR, Levenson VV. Methylation patterns in cell-free plasma DNA reflect removal of the primary tumor and drug treatment of breast cancer patients. International journal of cancer Journal international du cancer. 2011;128(2):492-9.
204. Keller A, Leidinger P, Lange J, Borries A, Schroers H, Scheffler M, et al. Multiple sclerosis: microRNA expression profiles accurately differentiate patients with relapsing-remitting disease from healthy controls. PloS one. 2009;4(10):e7440.
205. Koch MW, Metz LM, Kovalchuk O. Epigenetics and miRNAs in the diagnosis and treatment of multiple sclerosis. Trends in molecular medicine. 2013;19(1):23-30.
206. Waschbisch A, Atiya M, Linker RA, Potapov S, Schwab S, Derfuss T. Glatiramer acetate treatment normalizes deregulated microRNA expression in relapsing remitting multiple sclerosis. PloS one. 2011;6(9):e24604.
207. Otaegui D, Baranzini SE, Armananzas R, Calvo B, Munoz-Culla M, Khankhanian P, et al. Differential micro RNA expression in PBMC from multiple sclerosis patients. PloS one. 2009;4(7):e6309.

208. Moscarello MA, Mastronardi FG, Wood DD. The role of citrullinated proteins suggests a novel mechanism in the pathogenesis of multiple sclerosis. Neurochemical research. 2007;32(2):251-6.
209. Pedre X, Mastronardi F, Bruck W, Lopez-Rodas G, Kuhlmann T, Casaccia P. Changed histone acetylation patterns in normal-appearing white matter and early multiple sclerosis lesions. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2011;31(9):3435-45.
210. Wei G, Wei L, Zhu J, Zang C, Hu-Li J, Yao Z, et al. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. Immunity. 2009;30(1):155-67.
211. Wilson CB, Rowell E, Sekimata M. Epigenetic control of T-helper-cell differentiation. Nature reviews Immunology. 2009;9(2):91-105.
212. Janson PC, Linton LB, Bergman EA, Marits P, Eberhardson M, Piehl F, et al. Profiling of CD4+ T cells with epigenetic immune lineage analysis. J Immunol. 2011;186(1):92-102.
213. Lal G, Zhang N, van der Touw W, Ding Y, Ju W, Bottinger EP, et al. Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. J Immunol. 2009;182(1):259-73.
214. Cox MB, Cairns MJ, Gandhi KS, Carroll AP, Moscovis S, Stewart GJ, et al. MicroRNAs miR-17 and miR-20a inhibit T cell activation genes and are under-expressed in MS whole blood. PloS one. 2010;5(8):e12132.
215. Junker A, Krumbholz M, Eisele S, Mohan H, Augstein F, Bittner R, et al. MicroRNA profiling of multiple sclerosis lesions identifies modulators of the regulatory protein CD47. Brain. 2009;132(Pt 12):3342-52.
216. Du C, Liu C, Kang J, Zhao G, Ye Z, Huang S, et al. MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. Nat Immunol. 2009;10(12):1252-9.
217. Garcia-Manero G, Fenaux P. Hypomethylating agents and other novel strategies in myelodysplastic syndromes. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011;29(5):516-23.
218. Ghanem H, Cornelison AM, Garcia-Manero G, Kantarjian H, Ravandi F, Kadia T, et al. Decitabine can be safely reduced after achievement of best objective response in patients with myelodysplastic syndrome. Clinical lymphoma, myeloma & leukemia. 2013;13 Suppl 2:S289-94.
219. Kantarjian H, Oki Y, Garcia-Manero G, Huang X, O'Brien S, Cortes J, et al. Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. Blood. 2007;109(1):52-7.
220. Konac E, Varol N, Yilmaz A, Menevse S, Sozen S. DNA methyltransferase inhibitor-mediated apoptosis in the Wnt/beta-catenin signal pathway in a renal cell carcinoma cell line. Experimental biology and medicine. 2013;238(9):1009-16.

221. Krishnadas DK, Bao L, Bai F, Chencheri SC, Lucas K. Decitabine facilitates immune recognition of sarcoma cells by upregulating CT antigens, MHC molecules, and ICAM-1. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2014.
222. Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, Faderl S, et al. Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies. Blood. 2004;103(5):1635-40.
223. Issa JP, Gharibyan V, Cortes J, Jelinek J, Morris G, Verstovsek S, et al. Phase II study of low-dose decitabine in patients with chronic myelogenous leukemia resistant to imatinib mesylate. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2005;23(17):3948-56.
224. Camelo S, Iglesias AH, Hwang D, Due B, Ryu H, Smith K, et al. Transcriptional therapy with the histone deacetylase inhibitor trichostatin A ameliorates experimental autoimmune encephalomyelitis. Journal of neuroimmunology. 2005;164(1-2):10-21.
225. Ge Z, Da Y, Xue Z, Zhang K, Zhuang H, Peng M, et al. Vorinostat, a histone deacetylase inhibitor, suppresses dendritic cell function and ameliorates experimental autoimmune encephalomyelitis. Experimental neurology. 2013;241:56-66.
226. Zheng Q, Xu Y, Liu Y, Zhang B, Li X, Guo F, et al. Induction of Foxp3 demethylation increases regulatory CD4+CD25+ T cells and prevents the occurrence of diabetes in mice. Journal of molecular medicine. 2009;87(12):1191-205.
227. Fletcher JM, Lalor SJ, Sweeney CM, Tubridy N, Mills KH. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. Clin Exp Immunol. 2010;162(1):1-11.
228. de Jong EC, Smits HH, Kapsenberg ML. Dendritic cell-mediated T cell polarization. Springer Semin Immunopathol. 2005;26(3):289-307.
229. Cools N, Petrizzo A, Smits E, Buonaguro FM, Tornesello ML, Berneman Z, et al. Dendritic cells in the pathogenesis and treatment of human diseases: a Janus Bifrons? Immunotherapy. 2011;3(10):1203-22.
230. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol. 2013;31:563-604.
231. MacDonald KP, Munster DJ, Clark GJ, Dzionek A, Schmitz J, Hart DN. Characterization of human blood dendritic cell subsets. Blood. 2002;100(13):4512-20.
232. Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med. 2001;194(6):863-9.

233. Sarkar S, Fox DA. Dendritic cells in rheumatoid arthritis. Frontiers in bioscience : a journal and virtual library. 2005;10:656-65.
234. Darrasse-Jeze G, Deroubaix S, Mouquet H, Victora GD, Eisenreich T, Yao KH, et al. Feedback control of regulatory T cell homeostasis by dendritic cells in vivo. J Exp Med. 2009;206(9):1853-62.
235. Monrad S, Kaplan MJ. Dendritic cells and the immunopathogenesis of systemic lupus erythematosus. Immunol Res. 2007;37(2):135-45.
236. Lopez C, Comabella M, Al-zayat H, Tintore M, Montalban X. Altered maturation of circulating dendritic cells in primary progressive MS patients. Journal of neuroimmunology. 2006;175(1-2):183-91.
237. Hafler DA, Compston A, Sawcer S, Lander ES, Daly MJ, De Jager PL, et al. Risk alleles for multiple sclerosis identified by a genomewide study. The New England journal of medicine. 2007;357(9):851-62.
238. Gregory SG, Schmidt S, Seth P, Oksenberg JR, Hart J, Prokop A, et al. Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis. Nature genetics. 2007;39(9):1083-91.
239. Watanabe N, Wang YH, Lee HK, Ito T, Cao W, Liu YJ. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. Nature. 2005;436(7054):1181-5.
240. Hanabuchi S, Ito T, Park WR, Watanabe N, Shaw JL, Roman E, et al. Thymic stromal lymphopoietin-activated plasmacytoid dendritic cells induce the generation of FOXP3+ regulatory T cells in human thymus. J Immunol. 2010;184(6):2999-3007.
241. Hellings N, Baree M, Verhoeven C, D'Hooghe M B, Medaer R, Bernard CC, et al. T-cell reactivity to multiple myelin antigens in multiple sclerosis patients and healthy controls. J Neurosci Res. 2001;63(3):290-302.
242. Boven LA, Montagne L, Nottet HS, De Groot CJ. Macrophage inflammatory protein-1alpha (MIP-1alpha), MIP-1beta, and RANTES mRNA semiquantification and protein expression in active demyelinating multiple sclerosis (MS) lesions. Clin Exp Immunol. 2000;122(2):257-63.
243. Sorensen TL, Tani M, Jensen J, Pierce V, Lucchinetti C, Folcik VA, et al. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. The Journal of clinical investigation. 1999;103(6):807-15.
244. Chiurchiu V, Cencioni MT, Bisicchia E, De Bardi M, Gasperini C, Borsellino G, et al. Distinct modulation of human myeloid and plasmacytoid dendritic cells by anandamide in multiple sclerosis. Ann Neurol. 2013.
245. Schwab N, Zozulya AL, Kieseier BC, Toyka KV, Wiendl H. An imbalance of two functionally and phenotypically different subsets of plasmacytoid dendritic cells characterizes the dysfunctional immune regulation in multiple sclerosis. J Immunol. 2010;184(9):5368-74.

246. Sallusto F, Lanzavecchia A. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. Immunological reviews. 2000;177:134-40.
247. Sombekke MH, Lukas C, Crusius JB, Tejedor D, Killestein J, Arteta D, et al. HLA-DRB1*1501 and spinal cord magnetic resonance imaging lesions in multiple sclerosis. Arch Neurol. 2009;66(12):1531-6.
248. DeLuca GC, Ramagopalan SV, Herrera BM, Dyment DA, Lincoln MR, Montpetit A, et al. An extremes of outcome strategy provides evidence that multiple sclerosis severity is determined by alleles at the HLA-DRB1 locus. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(52):20896-901.
249. Aung LL, Fitzgerald-Bocarsly P, Dhib-Jalbut S, Balashov K. Plasmacytoid dendritic cells in multiple sclerosis: chemokine and chemokine receptor modulation by interferon-beta. J Neuroimmunol. 2010;226(1-2):158-64.
250. Migita K, Miyashita T, Maeda Y, Kimura H, Nakamura M, Yatsuhashi H, et al. Reduced blood BDCA-2+ (lymphoid) and CD11c+ (myeloid) dendritic cells in systemic lupus erythematosus. Clin Exp Immunol. 2005;142(1):84-91.
251. Richez C, Schaeverbeke T, Dumoulin C, Dehais J, Moreau JF, Blanco P. Myeloid dendritic cells correlate with clinical response whereas plasmacytoid dendritic cells impact autoantibody development in rheumatoid arthritis patients treated with infliximab. Arthritis Res Ther. 2009;11(3):R100.
252. Kalin JH, Butler KV, Kozikowski AP. Creating zinc monkey wrenches in the treatment of epigenetic disorders. Curr Opin Chem Biol. 2009;13(3):263-71.
253. Marks PA, Richon VM, Miller T, Kelly WK. Histone deacetylase inhibitors. Adv Cancer Res. 2004;91:137-68.
254. Xu WS, Parmigiani RB, Marks PA. Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene. 2007;26(37):5541-52.
255. Kim SC, Sprung R, Chen Y, Xu Y, Ball H, Pei J, et al. Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. Mol Cell. 2006;23(4):607-18.
256. Chung YL, Lee MY, Wang AJ, Yao LF. A therapeutic strategy uses histone deacetylase inhibitors to modulate the expression of genes involved in the pathogenesis of rheumatoid arthritis. Mol Ther. 2003;8(5):707-17.
257. Leoni F, Zaliani A, Bertolini G, Porro G, Pagani P, Pozzi P, et al. The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. Proc Natl Acad Sci U S A. 2002;99(5):2995-3000.
258. Reddy P, Sun Y, Toubai T, Duran-Struuck R, Clouthier SG, Weisiger E, et al. Histone deacetylase inhibition modulates indoleamine 2,3-dioxygenase-dependent DC functions and regulates experimental graft-versus-host disease in mice. J Clin Invest. 2008;118(7):2562-73.

259. Ge Z, Da Y, Xue Z, Zhang K, Zhuang H, Peng M, et al. Vorinostat, a histone deacetylase inhibitor, suppresses dendritic cell function and ameliorates experimental autoimmune encephalomyelitis. Exp Neurol. 2013;241:56-66.
260. Bosisio D, Vulcano M, Del Prete A, Sironi M, Salvi V, Salogni L, et al. Blocking TH17-polarizing cytokines by histone deacetylase inhibitors in vitro and in vivo. J Leukoc Biol. 2008;84(6):1540-8.
261. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med. 2001;193(2):233-8.
262. Xiao BG, Huang YM, Link H. Dendritic cell vaccine design: strategies for eliciting peripheral tolerance as therapy of autoimmune diseases. BioDrugs. 2003;17(2):103-11.
263. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med. 1992;176(6):1693-702.
264. Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods. 1999;223(1):77-92.
265. Butler LM, Agus DB, Scher HI, Higgins B, Rose A, Cordon-Cardo C, et al. Suberoylanilide hydroxamic acid, an inhibitor of histone deacetylase, suppresses the growth of prostate cancer cells in vitro and in vivo. Cancer Res. 2000;60(18):5165-70.
266. Cools N, Ponsaerts P, Van Tendeloo VF, Berneman ZN. Balancing between immunity and tolerance: an interplay between dendritic cells, regulatory T cells, and effector T cells. J Leukoc Biol. 2007;82(6):1365-74.
267. Serafini B, Rosicarelli B, Magliozzi R, Stigliano E, Capello E, Mancardi GL, et al. Dendritic cells in multiple sclerosis lesions: maturation stage, myelin uptake, and interaction with proliferating T cells. J Neuropathol Exp Neurol. 2006;65(2):124-41.
268. Jackson SH, Yu CR, Mahdi RM, Ebong S, Egwuagu CE. Dendritic cell maturation requires STAT1 and is under feedback regulation by suppressors of cytokine signaling. J Immunol. 2004;172(4):2307-15.
269. Jackson SH, Yu CR, Mahdi RM, Ebong S, Egwuagu CE. Dendritic cell maturation requires STAT1 and is under feedback regulation by suppressors of cytokine signaling. J Immunol. 2004;172(4):2307-15.
270. dos Santos AC, Barsante MM, Arantes RM, Bernard CC, Teixeira MM, Carvalho-Tavares J. CCL2 and CCL5 mediate leukocyte adhesion in experimental autoimmune encephalomyelitis--an intravital microscopy study. J Neuroimmunol. 2005;162(1-2):122-9.

271. Thewissen K, Nuyts AH, Deckx N, Van Wijmeersch B, Nagels G, D'Hooghe M, et al. Circulating dendritic cells of multiple sclerosis patients are proinflammatory and their frequency is correlated with MS-associated genetic risk factors. Mult Scler. 2014;20(5):548-57.
272. Sumpter TL, Thomson AW. The STATus of PD-L1 (B7-H1) on tolerogenic APCs. Eur J Immunol. 2011;41(2):286-90.
273. Wolfle SJ, Strebovsky J, Bartz H, Sahr A, Arnold C, Kaiser C, et al. PD-L1 expression on tolerogenic APCs is controlled by STAT-3. Eur J Immunol. 2011;41(2):413-24.
274. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124(4):783-801.
275. Schroder K, Sweet MJ, Hume DA. Signal integration between IFNgamma and TLR signalling pathways in macrophages. Immunobiology. 2006;211(6-8):511-24.
276. Roger T, Lugrin J, Le Roy D, Goy G, Mombelli M, Koessler T, et al. Histone deacetylase inhibitors impair innate immune responses to Toll-like receptor agonists and to infection. Blood. 2011;117(4):1205-17.
277. Chen L, Fischle W, Verdin E, Greene WC. Duration of nuclear NF-kappaB action regulated by reversible acetylation. Science. 2001;293(5535):1653-7.
278. Kaliszewska A, De Jager PL. Exploring the role of the epigenome in multiple sclerosis: a window onto cell-specific transcriptional potential. Journal of neuroimmunology. 2012;248(1-2):2-9.
279. Sospedra M, Martin R. Immunology of multiple sclerosis. Annu Rev Immunol. 2005;23:683-747.
280. Liggett T, Melnikov A, Tilwalli S, Yi Q, Chen H, Replogle C, et al. Methylation patterns of cell-free plasma DNA in relapsing-remitting multiple sclerosis. Journal of the neurological sciences. 2010;290(1-2):16-21.
281. Suarez-Alvarez B, Baragano Raneros A, Ortega F, Lopez-Larrea C. Epigenetic modulation of the immune function: a potential target for tolerance. Epigenetics : official journal of the DNA Methylation Society. 2013;8(7):694-702.
282. Slaets H, Hendriks JJ, Van den Haute C, Coun F, Baekelandt V, Stinissen P, et al. CNS-targeted LIF expression improves therapeutic efficacy and limits autoimmune-mediated demyelination in a model of multiple sclerosis. Molecular therapy : the journal of the American Society of Gene Therapy. 2010;18(4):684-91.
283. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3(7):RESEARCH0034.
284. Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, et al. Epigenetic control of the foxp3 locus in regulatory T cells. PLoS biology. 2007;5(2):e38.

285. Polansky JK, Kretschmer K, Freyer J, Floess S, Garbe A, Baron U, et al. DNA methylation controls Foxp3 gene expression. European journal of immunology. 2008;38(6):1654-63.
286. Wu CJ, Yang CY, Chen YH, Chen CM, Chen LC, Kuo ML. The DNA methylation inhibitor 5-azacytidine increases regulatory T cells and alleviates airway inflammation in ovalbumin-sensitized mice. International archives of allergy and immunology. 2013;160(4):356-64.
287. Herz J, Zipp F, Siffrin V. Neurodegeneration in autoimmune CNS inflammation. Experimental neurology. 2010;225(1):9-17.
288. Kehrmann J, Tatura R, Zeschnigk M, Probst-Kepper M, Geffers R, Steinmann J, et al. Impact of 5-Aza-2`-deoxycytidine and Epigallocatechin-3-gallate for induction of human regulatory T cells. Immunology. 2014.
289. Bettelli E, Korn T, Oukka M, Kuchroo VK. Induction and effector functions of T(H)17 cells. Nature. 2008;453(7198):1051-7.
290. Zhou L, Lopes JE, Chong MM, Ivanov, II, Min R, Victora GD, et al. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. Nature. 2008;453(7192):236-40.
291. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006;441(7090):235-8.
292. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. Nature. 2006;441(7090):231-4.
293. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity. 2006;24(2):179-89.
294. El-Behi M, Ciric B, Dai H, Yan Y, Cullimore M, Safavi F, et al. The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. Nat Immunol. 2011;12(6):568-75.
295. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. J Exp Med. 2006;203(10):2271-9.
296. Pette M, Fujita K, Kitze B, Whitaker JN, Albert E, Kappos L, et al. Myelin basic protein-specific T lymphocyte lines from MS patients and healthy individuals. Neurology. 1990;40(11):1770-6.
297. Jiang H, Chess L. Regulation of immune responses by T cells. The New England journal of medicine. 2006;354(11):1166-76.
298. Harbo HF, Lie BA, Sawcer S, Celius EG, Dai KZ, Oturai A, et al. Genes in the HLA class I region may contribute to the HLA class II-associated genetic susceptibility to multiple sclerosis. Tissue antigens. 2004;63(3):237-47.

299. Olerup O, Hillert J. HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation. Tissue antigens. 1991;38(1):1-15.
300. Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN. Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. European journal of immunology. 2001;31(4):1122-31.
301. Vukmanovic-Stejic M, Zhang Y, Cook JE, Fletcher JM, McQuaid A, Masters JE, et al. Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. The Journal of clinical investigation. 2006;116(9):2423-33.
302. Coquerelle C, Moser M. Are dendritic cells central to regulatory T cell function? Immunology letters. 2008;119(1-2):12-6.
303. Wakkach A, Fournier N, Brun V, Breittmayer JP, Cottrez F, Groux H. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. Immunity. 2003;18(5):605-17.
304. Yamazaki S, Iyoda T, Tarbell K, Olson K, Velinzon K, Inaba K, et al. Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. J Exp Med. 2003;198(2):235-47.
305. Bailey-Bucktrout SL, Caulkins SC, Goings G, Fischer JA, Dzionek A, Miller SD. Cutting edge: central nervous system plasmacytoid dendritic cells regulate the severity of relapsing experimental autoimmune encephalomyelitis. J Immunol. 2008;180(10):6457-61.
306. Vergelli M, Kalbus M, Rojo SC, Hemmer B, Kalbacher H, Tranquill L, et al. T cell response to myelin basic protein in the context of the multiple sclerosis-associated HLA-DR15 haplotype: peptide binding, immunodominance and effector functions of T cells. Journal of neuroimmunology. 1997;77(2):195-203.
307. Pashenkov M, Huang YM, Kostulas V, Haglund M, Soderstrom M, Link H. Two subsets of dendritic cells are present in human cerebrospinal fluid. Brain. 2001;124(Pt 3):480-92.
308. Teutsch SM, Booth DR, Bennetts BH, Heard RN, Stewart GJ. Identification of 11 novel and common single nucleotide polymorphisms in the interleukin-7 receptor-alpha gene and their associations with multiple sclerosis. Eur J Hum Genet. 2003;11(7):509-15.
309. Lundmark F, Duvefelt K, Iacobaeus E, Kockum I, Wallstrom E, Khademi M, et al. Variation in interleukin 7 receptor alpha chain (IL7R) influences risk of multiple sclerosis. Nature genetics. 2007;39(9):1108-13.
310. Hoe E, McKay FC, Schibeci SD, Gandhi K, Heard RN, Stewart GJ, et al. Functionally significant differences in expression of disease-associated IL-7 receptor alpha haplotypes in CD4 T cells and dendritic cells. J Immunol.184(5):2512-7.

311. Ito T, Wang YH, Duramad O, Hori T, Delespesse GJ, Watanabe N, et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. J Exp Med. 2005;202(9):1213-23.
312. Bernhard H, Disis ML, Heimfeld S, Hand S, Gralow JR, Cheever MA. Generation of immunostimulatory dendritic cells from human CD34+ hematopoietic progenitor cells of the bone marrow and peripheral blood. Cancer research. 1995;55(5):1099-104.
313. Figdor CG, de Vries IJ, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. Nature medicine. 2004;10(5):475-80.
314. Romani N, Gruner S, Brang D, Kampgen E, Lenz A, Trockenbacher B, et al. Proliferating dendritic cell progenitors in human blood. J Exp Med. 1994;180(1):83-93.
315. Nencioni A, Beck J, Werth D, Grunebach F, Patrone F, Ballestrero A, et al. Histone deacetylase inhibitors affect dendritic cell differentiation and immunogenicity. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007;13(13):3933-41.
316. Satpathy AT, Murphy KM, Kc W. Transcription factor networks in dendritic cell development. Seminars in immunology. 2011;23(5):388-97.
317. Dalod M, Chelbi R, Malissen B, Lawrence T. Dendritic cell maturation: functional specialization through signaling specificity and transcriptional programming. The EMBO journal. 2014;33(10):1104-16.
318. Zhang J, Cheng Y, Cui W, Li M, Li B, Guo L. MicroRNA-155 modulates Th1 and Th17 cell differentiation and is associated with multiple sclerosis and experimental autoimmune encephalomyelitis. Journal of neuroimmunology. 2014;266(1-2):56-63.
319. Zhu E, Wang X, Zheng B, Wang Q, Hao J, Chen S, et al. miR-20b Suppresses Th17 Differentiation and the Pathogenesis of Experimental Autoimmune Encephalomyelitis by Targeting RORgammat and STAT3. J Immunol. 2014;192(12):5599-609.
320. Suarez-Alvarez B, Rodriguez RM, Fraga MF, Lopez-Larrea C. DNA methylation: a promising landscape for immune system-related diseases. Trends in genetics : TIG. 2012;28(10):506-14.

Curriculum Vitae

Kristof Thewissen werd geboren op 5 mei 1986 te Bilzen. In 2005 behaalde hij zijn diploma Algemeen Secundair Onderwijs (ASO) in de studierichting Wiskunde (8u)-Wetenschappen aan het Heilig Graf Instituut te Bilzen. In datzelfde jaar startte hij zijn universitaire studies aan de Universiteit Hasselt/transnationale Universiteit Limburg (tUL) waar hij in 2007 zijn diploma Bachelor in de Biomedische Wetenschappen met onderscheiding haalde. Zijn masterjaar, in de afstudeerrichting klinische en moleculaire wetenschappen, voltooide hij eveneens aan de Universiteit Hasselt/tUL waar hij in 2009 afstudeerde met grote onderscheiding. Zijn eindwerk getiteld 'De rol van inhibitoire neurotransmitters op macrofaag functie tijdens neuroinflammatie' voerde hij uit in het Biomedische Onderzoeksinstituut (BIOMED) van de Universiteit Hasselt in de groep van Prof. Dr. Niels Hellings. Hij behaalde hiermee de eerste prijs tijdens de eindwerk posterpresentaties. In september 2009 startte hij zijn doctoraat aan BIOMED, na het behalen van een IWT-beurs. Zijn doctoraat was getiteld 'Het benutten van epigenetische mechanismen en dendritische cellen om multiple sclerose te behandelen'. Tijdens de hierop volgende jaren nam hij actief deel aan het onderwijs van de Universiteit Hasselt en volgde hij verschillende cursussen waaronder biosafety, good scientific conduct and labbook taking, Opinno entrepeneurship, BD Biosciences flow cytometry, masterclass MS pathologie en project management.

Bibliography

Publications

From this work

Thewissen K, Nuyts AH, Deckx N, Van Wijmeersch B, Nagels G, D'hooghe M, Willekens B, Cras P, Eijnde BO, Goossens H, Van Tendeloo VF, Stinissen P, Berneman ZN, Hellings N, Cools N.
Circulating dendritic cells of multiple sclerosis patients are proinflammatory and their frequency is correlated with MS-associated genetic risk factors.
Mult Scler. 2014 Apr;20(5):548-57

Thewissen K, Hendriks JJA, Vanhees M, Stinissen P, Slaets H, Hellings N.
Tolerogenic dendritic cells generated by in vitro treatment with SAHA are not stable in vivo.
Cell Transplantation, in revision

Thewissen K, Hendriks JJA, Stinissen P, Slaets H, Hellings N.
The DNA methylation inhibitor Decitabine completely blocks autoimmune mediated CNS demyelination.
Molecular Therapy, in revision

From collaborative projects

Carmans S, Hendriks JJ, **Thewissen K**, Van den Eynden J, Stinissen P, Rigo JM, Hellings N.
The inhibitory neurotransmitter glycine modulates macrophage activity by activation of neutral amino acid transporters.
J Neurosci Res. 2010 Aug 15;88(11):2420-30

Carmans S, Hendriks JJ, Slaets H, **Thewissen K**, Stinissen P, Rigo JM, Hellings N.
Systemic treatment with the inhibitory neurotransmitter γ-aminobutyric acid aggravates experimental autoimmune encephalomyelitis by affecting proinflammatory immune responses.
J Neuroimmunol. 2013 Feb 15;255(1-2):45-53

Raf Donders, Marjan Vanheusden, Jeroen FJ Bogie, Stylianos Ravanidis, **Kristof Thewissen**, Piet Stinissen, Wilfried Gyselaers, Jerome JA Hendriks and Niels Hellings.
Human Wharton's jelly-derived stem cells display immunomodulatory properties and transiently improve rat experimental autoimmune encephalomyelitis.
Submitted

Oral presentations

K. Thewissen, A. Nuyts, B. Van Wijmeersch, G. Nagels, M.B. D'hooghe, B. Wilekens, Zwi N. Berneman, P. Stinissen, V. Van Tendeloo, N. Cools, N. Hellings
Multiple sclerosis is associated with irregular numbers of circulating dendritic cells

- MS research days. 2010, Alphen aan de Rijn, Netherlands.
- European School of Neuroimmunology (ESNI). 2011, Glasgow, Scotland.
- PhD symposium; Cytokines and Cell Trafficking in Immunological Disorders. 2012, Hasselt, Belgium.

Kristof Thewissen, Jerome Hendriks, Piet Stinissen, Leen Slaets, Niels Hellings
Exploring a histone deacetylase inhibitor to generate tolerogenic dendritic cells for multiple sclerosis

- World Immune Regulation Meeting. 2014, Davos, Switzerland.

Poster presentations

K. Thewissen, A. Nuyts, B. Van Wijmeersch, G. Nagels, M.B. D'hooghe, B. Wilekens, Zwi N. Berneman, P. Stinissen, V. Van Tendeloo, N. Cools, N. Hellings
Multiple sclerosis is associated with irregular numbers of circulating dendritic cells

- Belgian Society for Cell and Developmental Biology (BSCDB). 2010, Hasselt, Belgium.
- Euron PhD days. 2010, Hasselt, Belgium.
- FWO-WOG MS symposium. 2010, Hasselt, Belgium.
- BIS meeting. 2011, Hasselt, Belgium.
- European Macrophage & Dendritic Cell Society (EMDS). 2011, Brussels, Belgium.
- FWO-WOG MS symposium. 2010, Brussels, Belgium

Kristof Thewissen, Jerome Hendriks, Piet Stinissen, Niels Hellings
Exploring a histone deacetylase inhibitor to generate tolerogenic dendritic cells for multiple sclerosis

- Euron PhD days. 2012, Maastricht, Netherlands.
- MS research days. 2012, Dorrwerth, Netherlands.
- FWO-WOG MS symposium. 2013, Antwerp, Belgium
- MS research days. 2013, Hasselt, Belgium.
- PhD symposium; cell-based therapies in central nervous system pathology. 2013, Hasselt, Belgium.

Oral presentation Award

Kristof Thewissen, Jerome Hendriks, Piet Stinissen, Leen Slaets, Niels Hellings
Exploring a histone deacetylase inhibitor to generate tolerogenic dendritic cells for multiple sclerosis

- World Immune Regulation Meeting. 2014, Davos, Switzerland.

Dankwoord

Het is zover! Na 4 ongelofelijke jaren kan ik eindelijk de laatste pagina's vullen van een geweldig avontuur. Terugkijkend op deze jaren, kan ik zeggen dat ik vele nieuwe ervaringen en inzichten heb opgedaan. Maar dit project had ik nooit tot een goed einde kunnen brengen zonder de steun en hulp die ik van iedereen gekregen heb. Ik ben misschien een man van weinig woorden, maar toch wil ik even de tijd nemen om kort iedereen te bedanken.

Eerst en vooral een woord van dank voor mijn promotor **Niels Hellings**. Dankzij hem begon ik als kersverse doctoraatsstudent in zijn 'T-cel clubje' mijn onderzoek rond jaja…dendritische cellen in MS. Een zeer interessant maar toch complex groepje cellen. Want hoe zat dat nu weer precies tussen immature vs mature DC en in welke categorie plaats je dan weer de tolerantie? Ondanks dat ik de enigste was die op dit celtype werkte in jouw groep, heb je met je ervaring het project altijd in de juiste richting kunnen sturen tijdens moeilijke momenten. Tijdens deze moeilijke periodes van experimenten die niet deden wat ze moesten, kon je ervoor zorgen dat ik terug gemotiveerd geraakte en geef nu toe wat zou een T-cel zijn zonder een DC. Toch ben ik blij dat je in de latere fase van mijn project mij de vrijheid hebt gegeven om deze DC'tjes aan de kant te schuiven zodat ik een nieuw idee, namelijk de rol van epigenetische behandeling in EAE, kon uitwerken. Hieruit is in mijn ogen het mooiste hoofdstuk van mijn doctoraat gekomen. Niet enkel op wetenschappelijk vlak heb ik veel bijgeleerd van jou, maar dankzij jou heb ik ook geleerd wat een congres nu precies boeiend maakt. Er zijn natuurlijk de vele interessante praatjes, maar het congres eten in Zwitserland zal mij altijd bijblijven ;-).

Piet Stinissen, als co-promotor heb jij ook een belangrijke rol gespeeld bij het tot stand komen van dit doctoraat. Tijdens onze jaarlijkse besprekingen zorgde jij altijd voor een interessante nieuwe kijk op de experimenten. Met je nuttige inbreng en tips heb je mij altijd dieper laten nadenken over het ruimere kader van mijn project.

Dan is het nu de beurt om mijn collega's van Antwerpen te bedanken, namelijk **Viggo Van Tendeloo**, **Nathalie Cools**, **Amber Nuyts**, **Wai Ping Lee** en

Nathalie Deckx. Jullie hebben mij altijd goed ontvangen in jullie labo. De mooie paper rond DC in MS is een mooi voorbeeld van de goede samenwerking met jullie onderzoeksgroep.

Ook wil ik heel graag mijn juryleden **Ivo Lambrichts**, **Veerle Somers**, **Jerome Hendriks**, **Leen Slaets**, **Zwi Berneman**, **Geert van Loo** en **Willem Voncken** bedanken voor het kritisch nalezen van mijn thesis en het aanleveren van constructieve suggesties wat het eindresultaat zeker verbeterd heeft. Speciale dank ook voor **Leen** die in de laatste jaren van het project altijd enthousiast was naar de resultaten van mijn onderzoek en meehielp aan de verdere uitwerking. Jouw inbreng gaf zeker een boost aan mijn project.

Dan ben ik nu eindelijk aanbeland aan mijn bureaucollega's waarmee ik de meeste uren heb doorgebracht. Eerst en vooral 'den ouwe' bureau**, Laura**, **Raf** en **silke**. Samen gestart aan ons doctoraat en samen vele mooie momenten meegemaakt die zorgden voor de nodige ontspanning tussen de experimenten door. **Laura**, jij stond altijd klaar met een luisterend oor en iemand met jouw optimisme ben ik nog niet vaak tegengekomen. Ik wens je nog veel geluk met je kleine spruit en je verdere carrière. **Silke**, ik heb altijd gedacht dat ik het grootste kookiemonster was (volgens mijn vrienden), maar als ik één ding van jouw geleerd heb dan is het wel dat je even graag, al is het misschien zelf meer, snoept dan mij. **Steven**, je hebt ons bureau wat later gejoined maar jouw idee van cakes on Thursday was een schot in de roos en samen met Raf zorgde jij voor de nodige humor. En dan ben ik aanbeland bij iemand die ik zeker niet mag vergeten, namelijk **Raf**. Tijdens mijn doctoraat ben jij toch wel de grootste steun geweest. Ik kon altijd bij je terecht voor weer eens te klagen over een experiment dat niet goed ging, maar vooral om te lachen en plezier te maken. En als we ooit eens afspreken om te gaan vissen, zullen we eerst nog eens de tutorial van Bill Dance bekijken ☺.

Nu ben ik aanbeland in 'den nieuwe' bureau met **Bieke**, **Tess**, **Marjan** en **Dana**. **Bieke**, de encyclopedie van ons bureau. Met de kennis die jij hebt, was er maar één adres als ik met een vraag zat. Je stond ook altijd klaar om te helpen. En in collaboratie met de andere dames **Tess** en **Marjan**, was het iedere morgen verschieten als ik het bureau opkwam en de vele foto's van acteurs op nuchtere maag te verwerken kreeg. Bedankt dames, dat ik nu ook de minder gekende

acteurs zoals Ryan Gosling heb leren kennen ;-P. Goed dat er dan nog steeds Jessica Alba als ruggensteuntje aanwezig was aan mijn helft. Enthousiaste **Tess**, ik ken geen opgewektere persoon als jouw en kan mijn geen dag inbeelden dat je niet met een lach binnenkwam. Ik kan met zekerheid zeggen dat jij voor de vrolijke noot zorgde op het bureau. **Marjan**, jouw ken ik al van toen ik nog les gaf aan je als student. Je kwam mij toen al voor als een hardwerkende student. Maar als collega op ons bureau werd dit al snel duidelijk. Je stond altijd klaar als ik met een vraag zat en samen met Tess bracht je leven in de brouwerij op ons bureau. **Dana**, de nieuwe aanwinst op ons bureau, ik heb je slechts enkele malen gesproken maar ben er van overtuigd dat je over alle vaardigheden beschikt om je doctoraat tot een succes te maken.

Naast de bureaucollega's zijn er nog een aantal personen die ik in het bijzonder wil bedanken en succes wensen in hun verdere carrière. **Stelios**, onze kameraad uit Griekenland, bedankt voor de ontspannende momenten tijdens onder andere de pokeravonden. **Jeroen**, ondanks dat je echt niet kunt pokeren, ben ik blij met zo'n goede onderzoeker op biomed als ik weer eens je expertise nodig had. Kokkin **Winde** die bij deze pokeravonden wel eens de maag van iedereen opvulde met lekkere spaghetti. **Kris** waarbij ik terecht kon met vragen over bepaalde experimenten zoals RT-PCR. **Nele** en **Ilse**, 2 toffe dames waarmee het altijd fijn is om kort even een babbeltje te slaan tussen mijn experimenten door.

Natuurlijk ook een dikke merci voor al de laboranten. **Igna** en **Christel** bij jullie kon ik altijd terecht met verschillende vragen. Bedankt voor al de hulp. In het bijzonder moet ik **Katrien** bedanken voor de vele hulp tijdens de dierproeven. Jouw helpende handen maakte het vele werk een stuk lichter. Ook merci aan **Paul**, **Joke** en **Eline** voor hun hulp in het animalium en het verzorgen van de dieren. **Veronique**, bedankt om ervoor te zorgen dat alles mooi in orde kwam voor de verdediging van mijn thesis.Als laatste wil ik ook alle andere **biomed collega's** bedanken die samen hebben gezorgd voor een leuke werksfeer.

Het **IWT** wil ik graag bedanken voor hun financiële ondersteuning van mijn project.

Naast mijn collega's wil ik nog een speciaal woordje richten aan mijn familie en vrienden. **Maarten**, **Tim** en **Kevin** waar moet ik beginnen over jullie. Een kort woordje zou al snel leiden tot een thesis op zich; Van de belevenissen in Praag tot het gezellig drinken van een pintje. Daarom zal ik hier houden tot een bedankje voor jullie steun, momenten van ontspanning en een welkome afleiding tijdens mijn doctoraat. Natuurlijk mag ik ook mijn vriendin **Nora** niet vergeten. We zijn nu iets meer dan een jaar samen en je hebt mij leren kennen in het laatste en drukste jaar van een doctoraat. Dit is natuurlijk soms niet gemakkelijk, maar toch tussen het geklaag over mislukte experimenten en het vele schrijfwerk door, heb je de tijd genomen om mij beter te leren en kennen. Jouw steun was zeker nodig tijdens deze laatste fase en nu het erop zit kunnen we ruim de tijd nemen om verder te werken aan de toekomst.
Last but not least wil ik my little sister, **Lynn,** bedanken voor de steun en de afleiding tijdens het schrijven. Misschien ben jij wel de enigste van de familie wat begrijpt wat ik allemaal precies heb uitgespookt in die 4 jaar. **Mama** en **papa**, zonder jullie was ik nooit zo ver gekomen! Jullie hebben mij de kans gegeven om te studeren en mij altijd vrij gelaten in mijn studiekeuzes. Bedankt voor jullie begrip en vertrouwen tijdens mijn doctoraat maar ook in de jaren daarvoor. Dankjewel voor alles!

Kristof, december 2014

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny…'." (Isaac Asimov)