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IMMUNOLOGICAL MECHANISMS REGULATING NEUROINFLAMMATION
A ROLE FOR C-TYPE LECTIN RECEPTORS AND VITAMIN D

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Immunological mechanisms regulating neuroinflammation
A role for C-type lectin receptors and vitamin D

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By

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“Warwaraan deh gnaka bena tanka terewouko dem yonam” *

– Senegalese proverb

“Tchimbe red, pa moli” **

– Créole proverb

“Quand on ne trouve pas ce qu'on cherche, il arrive qu'on trouve beaucoup mieux” ***

– French proverb

*The centipede does not stop for one leg

** Stay strong, do not falter

*** When you do not find what you are looking for, you sometimes find something better

To my family

ABSTRACT

Autoimmunity of the central nervous system (CNS) such as in Multiple Sclerosis (MS) or its animal model Experimental Autoimmune Encephalomyelitis (EAE) requires the activation of self-reactive immune cells and the presentation of self-antigen by mature antigen presenting cells (APCs).

In this thesis I have studied genetic and environmental mechanisms affecting APCs and CD4+ T cells which regulate susceptibility to EAE induced with myelin oligodendrocyte glycoprotein (MOG) in the rat.

Bone marrow derived dendritic cells (BMDCs) are frequently used as *in vitro* alternatives to APCs, particularly DCs observed *in vivo*. However it is crucial to discriminate which cell type is being generated as they will serve different functions. In paper I, we have first phenotypically and functionally characterized different types of *in vitro* generated BMDCs with different protocols. We have shown that BMDCs generated with FMS-like tyrosine kinase 3 ligand (FL-BMDCs) shared gene expression and characteristics of classical DCs observed in the body. We also demonstrated that BMDCs generated with the cytokines GM-CSF and IL-4 (G4-BMDCs) were pro-inflammatory and resembled monocytes derived DCs (MCs) generated *in vivo* under inflammatory conditions.

In the EAE model, APCs are crucial for the priming of CD4+ T cells in the draining lymph nodes following immunization, as well as their reactivation in the CNS prior to disease onset. In paper II, we have characterized how two c-type lectin receptors (CLRs), MCL and Mincle, conferred susceptibility to EAE by directing the re-activation of CD4+ T cells in the CNS towards the pathogenic IL-17-producing T helper (Th17) phenotype. We showed that this was in part due to the alarmin SAP130, an endogenous ligand of MCL and Mincle. Furthermore, we determined that the MCL/MINCLE signaling pathway was hyperactive in MS blood monocytes and might contribute to disease severity.

Many environmental factors have been linked to increased risk of developing MS including lack of sun exposure and vitamin D deficiency. In paper III, we described how vitamin D affects the activation of CD4+ T cells via epigenetic mechanisms (miRNA, DNA methylation and histone modification) resulting in a reduction of their pathogenic potential.

This thesis showed, using different approaches in rodent models and human material, how genetic and environmental factors relevant for MS could regulate susceptibility to EAE by modulating the development of pathogenic Th17 cells. In paper I, we characterized BMDCs routinely used *in vitro* to study immune functions of myeloid cells. In paper II and paper III, we addressed two distinct mechanisms regulating T cell activation and EAE development. This work presented integrative strategies to address biological questions and provided novel insight into the immunological aspects of autoimmune neuroinflammation.

LIST OF SCIENTIFIC PAPERS

I. N'DIAYE M, Warnecke A, Flytzani S, Abdelmagid N, Ruhrmann S, Olsson T, Jagodic M, Harris RA, Guerreiro-Cacais AO.
Rat bone marrow-derived dendritic cells generated with GM-CSF/IL-4 or FLT3L exhibit distinct phenotypical and functional characteristics
Journal of Leukocyte Biology, 2016, volume 99 no. 3, page 437-446

II. N'DIAYE M, Flytzani S, Brauner S, Warnecke A, Adzemovic MZ, Kular L, Piket E, Mela F, Choi HY, Magg V, James T, Linden M, Reichardt H, Daws MR, Fossum S, Kockum I, Harris RA, Olsson T, Guerreiro-Cacais AO, Jagodic M.
The C-type lectin receptors MCL and Mincle regulate susceptibility to experimental neuroinflammation and associate with disease activity in Multiple Sclerosis
Manuscript

III. Zeitelhofer M, Adzemovic MZ, Gomez-Cabrero D, Bergman P, Hochmeister S, N'DIAYE M, Paulson A, Ruhrmann S, Almgren M, Tegnér JN, Ekström TJ, Guerreiro-Cacais AO, Jagodic M.
Functional genomics analysis of vitamin D effects on CD4+ T cells *in vivo* in experimental autoimmune encephalomyelitis
Proceedings of the National Academy of Sciences of the United States of America (PNAS), 2017, volume 114 no. 9, page 1678–1687

PUBLICATIONS NOT INCLUDED IN THIS THESIS

1. Nitration of MOG diminishes its encephalitogenicity depending on MHC haplotype.
   Warnecke A, Musunuri S, N'diaye M, Sandalova T, Achour A, Bergquist J, Harris RA. J Neuroimmunol. 2017 Feb 15;303:1-12.
   doi: 10.1016/j.jneuroim.2016.11.008. Epub 2016 Nov 26.

2. Translational utility of experimental autoimmune encephalomyelitis: recent developments.
   Guerreiro-Cacais AO, Laaksonen H, Flytzani S, N'diaye M, Olsson T, Jagodic M. J Inflamm Res. 2015 Nov 13;8:211-25. doi: 10.2147/JIR.S76707. eCollection 2015. Review.

3. MOG-induced experimental autoimmune encephalomyelitis in the rat species triggers anti-neurofascin antibody response that is genetically regulated.
   Flytzani S, Guerreiro-Cacais AO, N'diaye M, Lindner M, Linington C, Meinl E, Stridh P, Jagodic M, Olsson T. J Neuroinflammation. 2015 Oct 29;12:194. doi: 10.1186/s12974-015-0417-2.

CONTENTS

LIST OF ABBREVIATIONS

MS	Multiple sclerosis
EAE	Experimental autoimmune encephalomyelitis
DCs	Dendritic cells
APCs	Antigen presenting cells
MHC	Major histocompatibility complex
pDCs	Plasmacytoid DCs
cDCs	Classical DCs
IFN	Interferon
SLE	Systemic lupus erythematous
CNS	Central nervous system
CSF	Cerebrospinal fluid
MHC-II	Major histocompatibility complex class II
Th	T helper
MHC-I	Major histocompatibility complex class I
Tregs	Regulatory T cells
TCR	T cell receptor
TNF	Tumor necrosis factor
BBB	Blood-brain barrier
BCB	Blood-CSF barrier
PAMPs	Pattern-associated molecular patterns
PRRs	Pattern recognition receptors
DAMPs	Damage-associated molecular patterns
TLRs	toll-like receptors
CLRs	C-type lectin receptors
DA	Dark agouti
PVG	Piebold-viral-glaxo
MBP	Myelin basic protein

mRNA	Messenger RNA
siRNA	Small interfering RNA
qPCR	Quantitative polymerase chain reaction
ELISA	Enzyme-linked immunoabsorbent assay
BMDCs	Bone marrow derived dendritic cells
MRI	Magnetic resonance imaging
CIS	Clinically isolated syndrome
RRMS	Relapsing remitting multiple sclerosis
SPMS	Secondary progressive multiple sclerosis
PPMS	Primary progressive multiple sclerosis
GWAS	Genome wide association studies
HLA	Human leukocyte antigen
SNP	Single nucleotide polymorphisms
VDR	Vitamin D receptor
aHSCT	Autologous hematopoietic stem cell transplantation
CFA	Complete Freund’s adjuvant
IFA	Incomplete Freund’s adjuvant
PTX	Pertussis toxin
MOG	Myelin oligodendrocyte glycoprotein
GFP	Green fluorescent protein

1. Introduction

Research on basic immunology, Multiple Sclerosis (MS) and its animal model Experimental Autoimmune Encephalomyelitis (EAE) is very active and knowledge continues to expand every day, leading to an extensive amount of information. The goal of this section is to provide the reader with sufficient background knowledge in order to understand the work done in this thesis.

I. The immune system

The role of the immune system is to protect us from disease, such as infection and cancer. Our body contains a tightly regulated network of cells whose sole purpose is to defend us against extrinsic attacks and intrinsic hazards (Fig. 1).

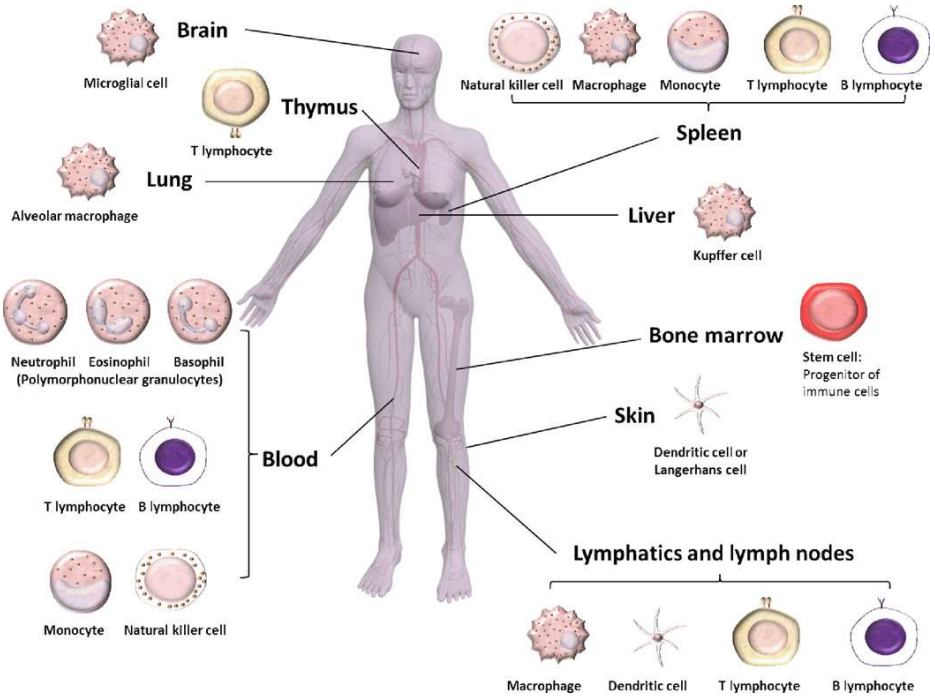

Fig. 1 Human body showing the various organs of the immune system and the distribution of the various immune cells into the immune organs and other organs that are rich in macrophages. Mahmoud Elsabahy and Karen L. Wooley, "Cytokines as biomarkers of nanoparticle immunotoxicity", Chemical Society Reviews 2013;42:5552-5576[1]

A normal immune response has an inflammatory phase (to remove the threat) followed by the resolution of the inflammation and an anti-inflammatory phase (to enable tissue repair and return of physiological functions). The regulation of an immune response occurs in space and time to prevent damage to adjacent non-affected tissue or prolonged damage to the affected organ (as occurring during chronic inflammation).

However, dysregulation of the immune system can cause disease itself, such as allergy or autoimmunity. Allergy is a hypersensitivity reaction of the immune system to antigens that do not generate immune responses in most people (e.g. food or pollen allergies, asthma). Autoimmunity is an aberrant response of the immune system to self-antigen, resulting in organ dysfunction and possibly death (e.g. type 1 diabetes, rheumatoid arthritis, multiple sclerosis).

The components of the immune system can be separated into two parts: the innate and the adaptive immune systems. Both parts are complementary arms of inflammation and can contribute to different phases of the immune response (initiation, propagation and resolution).

a) Cytokine network

Cytokines are soluble signaling molecules that regulate many bodily functions during homeostasis through promoting communication between immune and non-immune cells[2]. During inflammation, activation of innate immune cells induces the production of a specific cytokine signature that will dictate the subsequent adaptive immune response. Cytokines influence the expression of other cytokines and can cause, if not tightly regulated, a cytokine storm or cytokine cascade that can be potentially fatal for the host, as evident in cases of sepsis.

b) The innate immune system

The innate immune system developed earlier during evolution than the adaptive immune system and includes cells capable of sensing infection and tissue damage [3-5]. These cells are also crucial in shaping the subsequent adaptive immune response. The innate immune system comprises natural killer cells, innate lymphoid cells, mucosal associated invariant T cells, basophils, eosinophils, mast cells as well as phagocytic cells such as dendritic cells (DCs), macrophages, monocytes and neutrophils. The work of this thesis mainly focuses on the role of phagocytic innate immune cells and they will be described further in the next section.

(i) Peripheral innate immune system: dendritic cells, neutrophils, tissue monocytes and macrophages

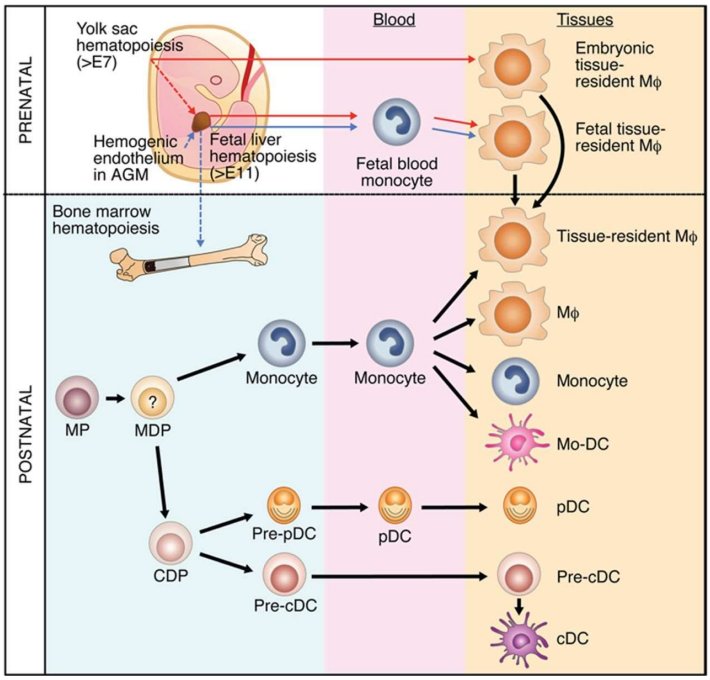

Fig. 2 Macrophages and dendritic cells ontogeny. Myron I. Cybulsky, Cheolho Cheong and Clinton S. Robbins, "Macrophages and Dendritic Cells Partners in Atherogenesis", Circulation Research. 2016;118:637-652[6]

The passage of fluids between the blood, tissue and lymph allows the circulation of nutrients to the tissue and the drainage of waste and microorganisms. Blood, interstitial fluid and lymph can collect and transport pathogens to the lymphoid organs, i.e. lymph nodes and spleen, where antigen presenting cells (APCs) can capture and present antigens to lymphocytes. Moreover, APCs are also present in abundance in barrier tissues such as the skin and mucosa (gut, nose, lungs) where they can form discrete structures of lymphoid tissue named skin, mucosal or nasal -associated lymphoid organs (SALT, MALT, NALT respectively) or migrate to the lymphoid organs. Thereafter, APCs can interact with lymphocytes and shape an appropriate immune response to pathogens or respond to damage.

Dendritic cells are the most potent of all the APCs and constitutively express the surface protein major histocompatibility complex (MHC) class II[7]. Their name arises from their star shape, with lots of protruding extensions or dendrites that enable them to sample the environment for dangers such as pathogens or damaged cells. While the existence of DCs is well accepted since their discovery by Ralph Steiman in 1973[8] (for which he was awarded a Nobel prize in 2011), distinguishing DCs from other phagocytic cells has always been a challenge as many markers that were thought to be DC-specific (e.g. CD11c) proved to be expressed by other immune cells in both health and disease states. A new nomenclature based on ontogeny allowed for the distinction of true DCs from monocyte-derived dendritic-looking cells[9]. DCs arise from bone marrow hematopoietic stem cells from a distinct common dendritic precursor, and can be divided into two subclasses: plasmacytoid DCs (pDCs) and classical DCs (cDCs) [10-13] (Fig. 2). pDCs are present in the blood as well as in lymphoid organs and their morphology is more similar to that of plasma cells. They are a major source of type I interferon (IFN) and are crucial for antiviral responses[14-16]. They can also greatly contribute to autoimmune disease with strong type I IFN signatures such as in systemic lupus erythematous (SLE)[17]. Pre-cDCs exit the bone marrow and populate the tissue and secondary lymphoid organs where they differentiate into immature cDCs[10,11]. Once activated by danger signals, mature tissue DCs upregulate CCR7 and migrate to the draining lymph nodes where they activate cells of the adaptive immune system[18,19]. DCs residing in the spleen and lymph nodes can survey for danger signals present in body fluids (blood, lymph)[20].

Neutrophils are short-lived phagocytes that egress from the bone marrow fully mature and circulate in the blood[21]. They are one of the first immune cells recruited by local APCs to the sites of infection or damage. They are potent effector cells that can cause great tissue damage if not tightly regulated. Indeed, once activated they can release cytokines[22,23], proteases[24] and reactive oxygen species[25] or generate extracellular traps[26,27] (netosis) in order to destroy the invading pathogens and to propagate inflammation. Apoptotic dying neutrophils facilitate the resolution of inflammation by preventing the further recruitment of neutrophils and promoting their clearance by macrophages[28,29].

Monocytes are generated in the bone marrow and migrate to the blood (Fig. 2). They can be separated into two main subsets, classical and non-classical monocytes, in human (CD14+ and $CD14^{low}$ CD16+), mouse ($LY6C^{hi}$ CCR2+ CD62L+ $CX3CR1^{mid}$ and $LY6C^{low}$ CD43+ $CX3CR1^{hi}$) and rats ($CCR2^{hi}$ $CX3CR1^{low}$ $CD43^{low}$ and $CCR2^{low}$ $CX3CR1^{hi}$ $CD43^{hi}$)[30,31]. Monocytes do not contribute to the majority of tissue macrophage pools except in some specific areas such as the gut, skin,

pancreas, heart, choroid plexus and possibly liver[32,33]. However, they are rapidly recruited to inflammatory sites where they can differentiate into macrophages or dendritic-like cells and contribute to inflammation[34] (Fig. 3). Non-classical monocytes patrol the blood vessels and are important for vascular repair, and are also thought to play a role in the resolution of inflammation by promoting wound healing[35,36] (Fig. 3).

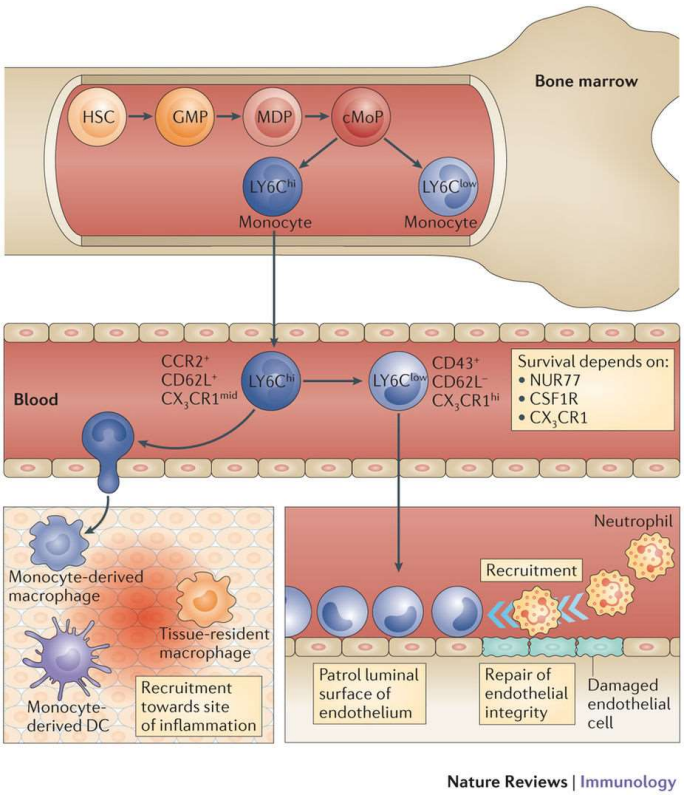

Fig. 3 The mouse monocyte compartment. Florent Ginhoux and Steffen Jung, "Monocytes and macrophages: developmental pathways and tissue homeostasis", Nature review Immunology 2014;14:392–404[37]

Macrophages are present throughout the whole body, colonizing the different tissues during embryonic development and early life (Fig. 2)[38-44]. The organ environment will affect resident macrophages in adopting certain shapes and functions that are necessary for tissue maintenance and homeostasis (Fig. 4). For instance, osteoclasts play a crucial role in bone remodeling[45] and alveolar macrophages are necessary for pulmonary surfactant catabolism[46]. Once seeded throughout the body most macrophage subsets are able to self-renew and maintain their numbers during homeostasis with little or no contribution from blood monocytes[33,47].

In response to threats, local APCs (macrophages or DCs) as well as patrolling non-classical monocytes will be activated and recruit neutrophils to the site of insult by secreting chemokines (CXCL1, CXCL2). The inflammatory environment will activate the neutrophils and lead to the recruitment of more immune cells such as classical inflammatory monocytes. Time or the clearance of danger will lead to the death of neutrophils and other infiltrating immune cells by apoptosis, which will in turn facilitate the resolution of inflammation and tissue repair[48] (Fig. 5).

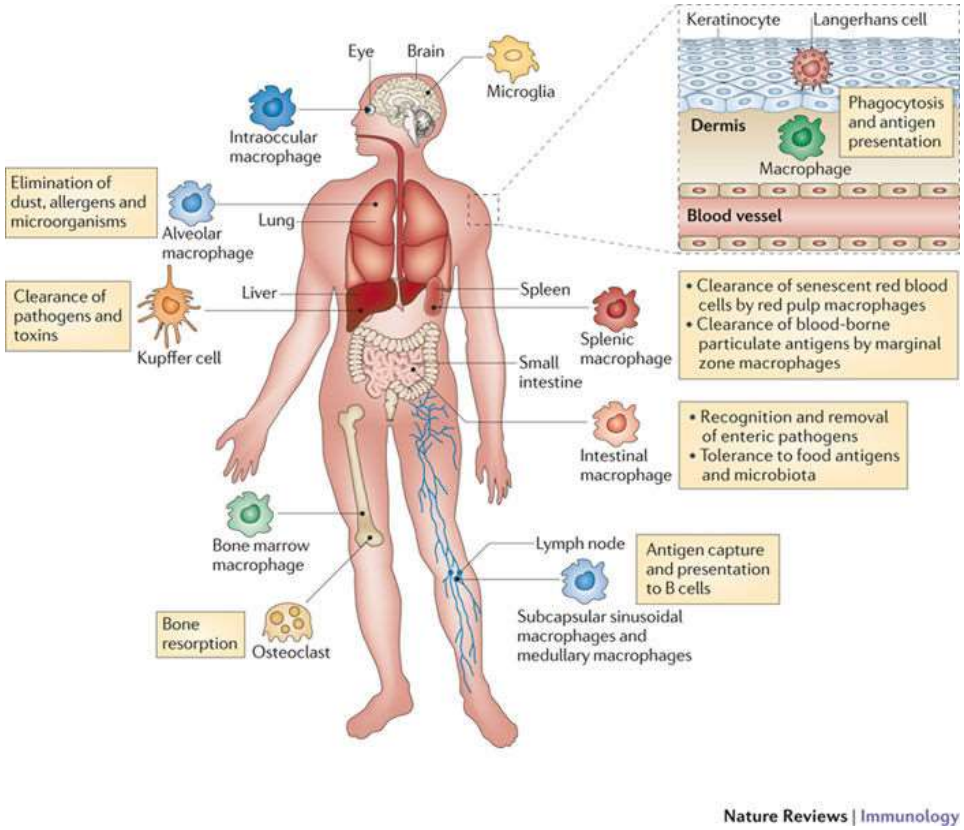

Fig. 4 Tissue macrophages perform important homeostatic functions. Peter J. Murray and Thomas A. Wynn, "Protective and Pathogenic Functions of Macrophage Subsets", Nature review immunology, 2001;11:723–737[49]

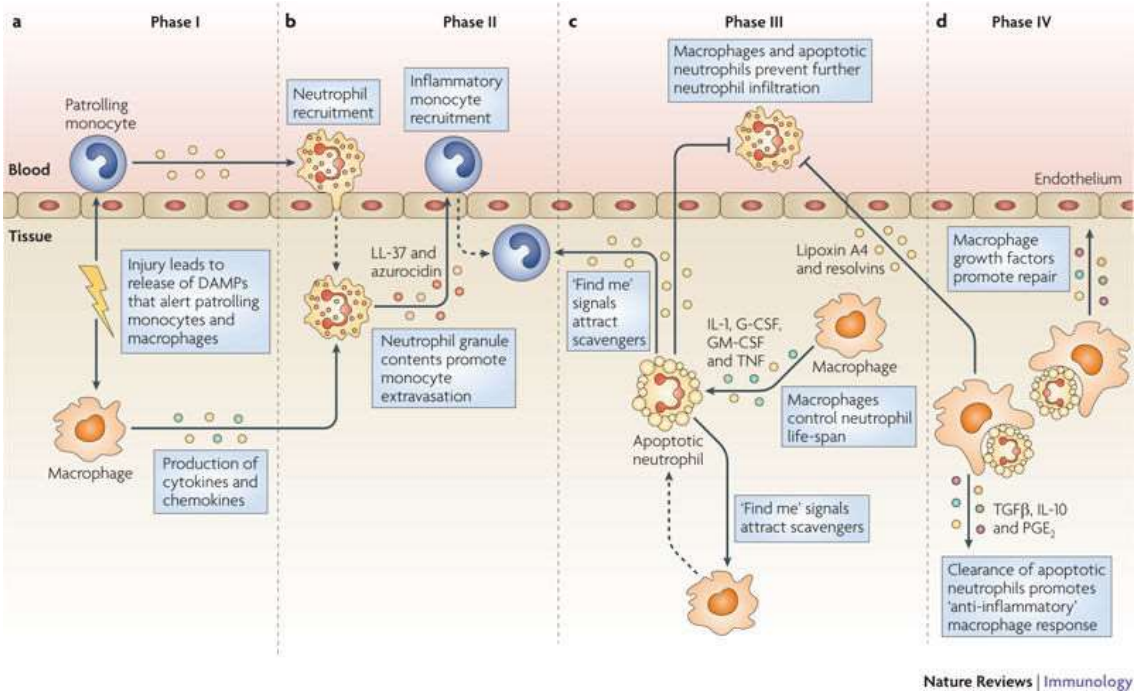

Fig. 5 Phagocyte interactions in inflammation. Oliver Soehnlein and Lennart Lindbom, "Phagocyte partnership during the onset and resolution of inflammation", Nature reviews Immunology 2010;10:427–439[48]

(ii) Microglia and Meningeal/Perivascular/Choroid plexus Macrophages

Microglia are the resident macrophages of the central nervous system (CNS) parenchyma (Fig. 6). Like other macrophages, they are the first myeloid cells seeded in the CNS during embryogenesis and can self-renew *in situ* without contribution from blood monocytes[41]. They are important for CNS development, helping to shape the neuronal circuit[50,51]. During post-natal life they monitor the environment for danger and support neuronal functions[52,53]. Resting microglia have a highly ramified shape with long processes which allow them to monitor their surroundings. Once activated, they assume an amoeboid shape and can promote inflammation which, if not controlled, can contribute to tissue damage.

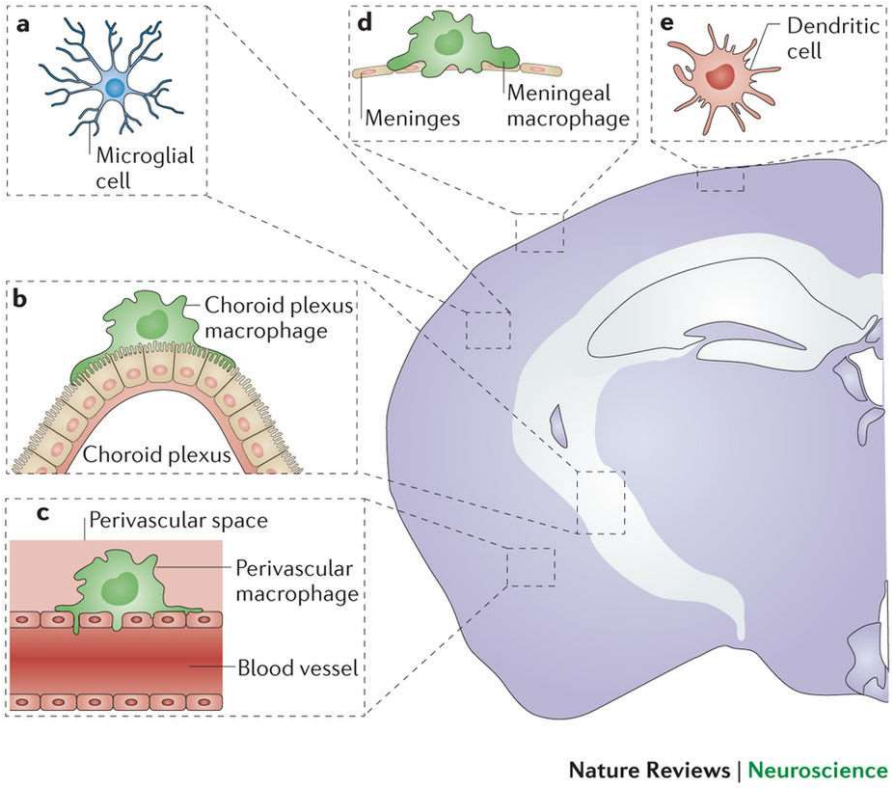

Fig. 6 Myeloid cell types in the CNS. Marco Prinz and Josef Priller, "Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease", Nature review neuroscience, 2014; 15:300–312[54]

Non-parenchymal macrophages are present within the meninges, around the blood vessels and in the choroid plexus [55](Fig. 6). These macrophages are strategically placed between the cerebrospinal fluid (CSF) and blood vessels or at the interface of the interstitial fluid and blood in order to monitor and respond to events within the CNS while also responding to peripheral influences[55]. Similar to other macrophages, they can also express MHC class II (MHC-II) once activated and are therefore capable of presenting antigens to T cells[56].

c) The adaptive immune system

While the innate immune system provides a first line of defense against threats, the activation of the adaptive immune system is crucial to provide highly specific and long-lasting immunological memory.

(i) The T lymphocytes

T cells are lymphocytes that develop in the bone marrow but mature in the thymus. They can be separated into three functional subsets, (a) helper T cells, (b) cytotoxic T cells and (c) regulatory T cells. As this thesis mainly focuses on the role of T helper (Th) cells, I will briefly introduce the two other subsets before focusing on Th cells.

Cytotoxic T cells mainly express the CD8 marker, although there have been recent reports of $CD4^+$ cytotoxic lymphocytes. Their role is to directly kill cells that they recognize via the MHC class I (MHC-I) for $CD8^+$ cells or MHC-II for $CD4^+$ cells[57]. This is the case for virally infected cells or tumor cells, for example. On the contrary, regulatory T cells (Tregs) are mostly known as $CD4^+$ Tregs but can also be $CD8^+$ cells. There are 2 main subsets of Tregs, those that arise directly from thymic selection named thymic Tregs (tTregs), and those that arise from peripheral activation named induced pTregs. *In vitro* induced Tregs are referred to as iTregs. Regulatory T cells are important to regulate immune responses as they exert immunosuppressive actions through different mechanisms[58].

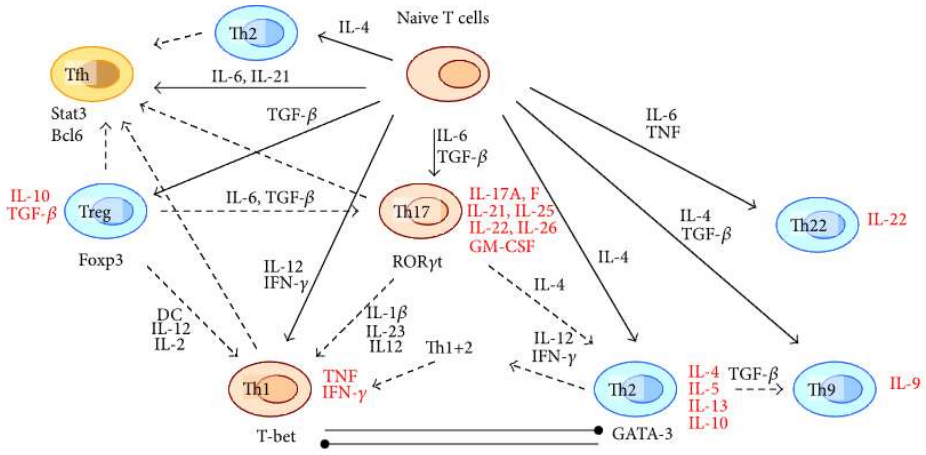

Fig. 7 Simplified scheme of T cell differentiation pathways and plasticity (dashed arrows). Ekaterina A. Ivanova and Alexander N. Orekhov, "T Helper Lymphocyte Subsets and Plasticity in Autoimmunity and Cancer: An Overview", BioMed Research International, 2015[59]

T helper lymphocytes, mainly $CD4^+$ cells, are at the heart of the adaptive immune response. They are crucial for relaying the information provided by the activated APCs in order to produce an appropriate adaptive response to danger[60]. Absence of T helper cells represents a major limitation in our defense against pathogens, as evident in HIV patients affected by AIDS[61]. In a nutshell, three steps are necessary for the activation of $CD4^+$ T cells. Signal 1: mature APCs present antigen in the context of MHC-II that will be recognized by the T cell receptor (TCR). Signal 2: mature APCs upregulate co-stimulatory molecules (e.g. CD80, CD86) that can be recognized by receptors on the T cells (e.g. CD28). Signal 3: mature APCs secrete a pattern of cytokines specific for the danger encountered that will determine the type of Th cell the activated T cell will become[62,63] (Fig. 7).

Th1 and Th17 lymphocytes are two pro-inflammatory subsets of Th cells. Th1 cells are important for the response to intracellular bacteria such as *Mycobacteria tuberculosis*, while Th17 cells are crucial for anti-fungal immunity (e.g. *Candida albicans*). Th1 cells are induced

by the cytokines IL-12 and IFNγ via the transcription factor Tbet and can produce tumor necrosis factor (TNF) and IFNγ that will boost macrophage activation and reactive oxygen species production[64,65]. Th17 cells are induced by IL-1, IL-6 and TGFβ via the transcription factor Rorγt[66,67]. They produce the cytokines IL-17, IL-21 and IL-22 that will boost the activation of immune and non-immune cells as well as neutrophil recruitment[68,69]. Both subsets have been implicated in autoimmunity and since most of the knowledge about their role arises from animal studies they will be discussed in more detail in section III.

(ii) The B Lymphocytes

B cells are the effectors of the humoral branch of adaptive immunity. Their main role is to provide long-term protection against pathogens by secreting specific antibodies. B cells are also very good antigen presenting cells and producers of cytokines. The work of this thesis focuses on the interaction of T cells with APCs, but B lymphocytes seem to also be key players in multiple sclerosis pathogenesis[70].

d) CNS immunity

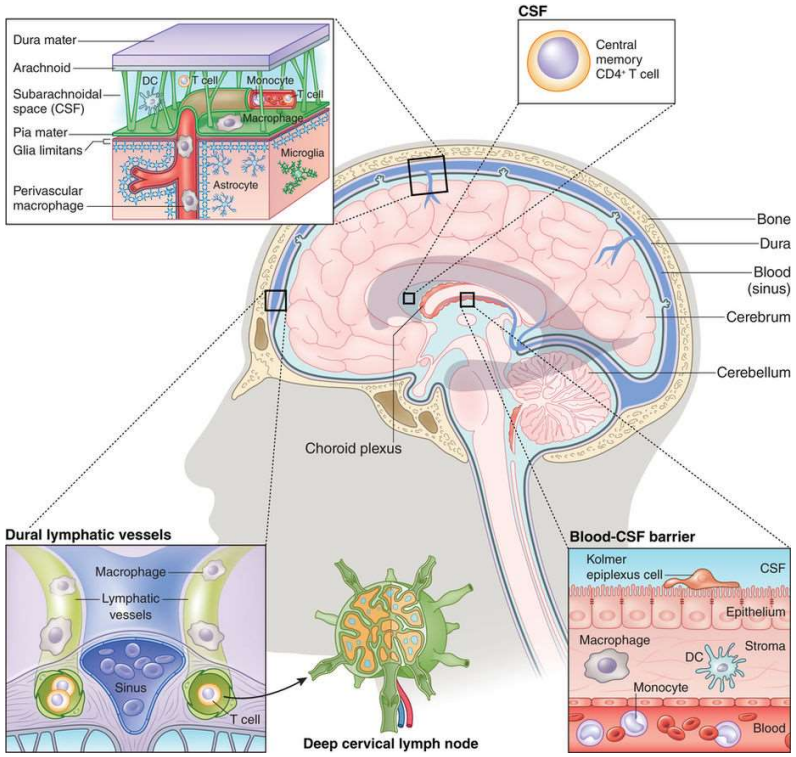

Fig. 8 The CNS immune system during homeostasis. Prinz M and Priller J, "The role of peripheral immune cells in the CNS in steady state and disease", Nature review Neuroscience 2017;20:136–144[71]

For a long time the CNS was considered to be 'immune privileged' due to its inability to reject grafts and because it is protected from immune invasion by a semi-permeable multi-layered barrier that limits the entry of cells and molecules from the blood, namely the blood-brain or blood-CSF barrier (BBB and BCB, respectively). However, macrophages and dendritic cells, as well as other innate immune cells, populate different areas of the CNS, including the meninges, in steady-state, and memory/activated T cells patrol the CNS during homeostasis[71-

[73]. Furthermore, the CNS is capable of sensing and responding to peripheral inflammation, resulting in resident cell activation and cytokine production[74]. Finally, CNS antigens can be found in deep cervical lymph nodes either traveling via the dural or nasal lymphatics[75-78]. Therefore, all the elements are in place to enable both innate and adaptive immune responses within the CNS (Fig. 8).

e) PAMPs, DAMPs and PRRs

Invading pathogens can be recognized by the immune system through conserved motifs named pattern-associated molecular patterns (PAMPs). Cells of the immune system, mostly innate immune cells, express receptors named pattern recognition receptors (PRRs) capable of recognizing such motifs. In an ideal scenario, recognition of PAMPs by PRRs results in cell activation and induces an inflammatory response specific for the type of pathogen recognized, which is ultimately eliminated. PRRs can also recognize endogenous molecules that are released by activated, stressed or damaged cells called danger/damage associated molecular patterns (DAMPs). These molecules are capable of inducing an inflammatory response in the absence of infectious pathogens, a process referred to as sterile inflammation[79,80].

There are many types of PRRs including toll-like receptors (TLRs), retinoic acid-inducible gene I -like receptors, nucleotide-binding leucine-rich repeat containing receptors and C-type lectin receptors (CLRs). While the most studied family of PRRs is probably the TLRs, the focus of this thesis is on CLRs. This large superfamily contains over a thousand members and, despite having been discovered over 100 years ago, only recently has there been an increased interest in their investigation[81]. We have been particularly interested in two CLRs of the Dectin-2 cluster, macrophage C-type lectin (MCL, Clec4d) and macrophage inducible C-type lectin (Mincle, clec4e), which can be found on human chromosome 12, mouse chromosome 6 and rat chromosome 4[82,83] (Fig. 9).

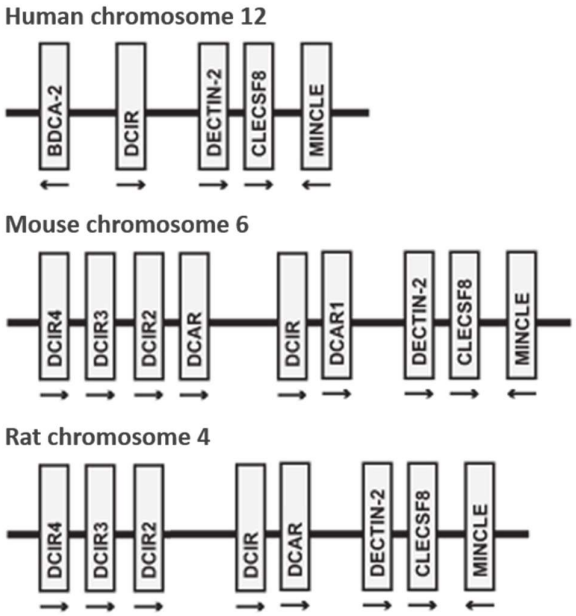

Fig. 9 The Dectin-2 cluster of C-type lectins. Adapted from Kerscher[82] B, Willment JA and Brown GD, "The Dectin-2 family of C-type lectin-like receptors: An update", International Immunology 2013;25(5):271-277.

Members of the Dectin-2 cluster also include BDCA-2, DCAR, Dectin2 and DCIR. These receptors are primarily expressed by antigen presenting cells of myeloid origin. Different PRRs induce different signaling cascades and gene inductions; Figure 10 shows a schematic comparing signaling of CLRs to TLRs[84].

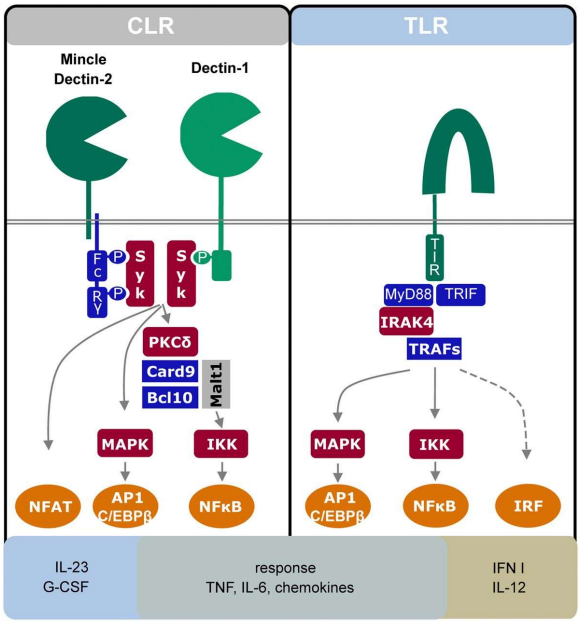

Fig. 10 Schematic comparison of CLR and TLR signaling. Jenny Ostrop and Roland Lang, "Collaboration, and Conflict: Signal Integration of Syk-Coupled C-Type Lectin Receptors", Journal of Immunology 2017;198(4):1403-1414[84]

Members of the Dectin-2 cluster are activating receptors which signal via FCRγ, Syk, and CARD9-BCL10-MALT1 to induce NF-κB activation (Fig. 11). Moreover, the syk-CARD9 pathway of CLR signaling induces the production of IL-1β, IL-6 and IL-23 by immune cells that can in turn promote the differentiation of T cells to the Th17 phenotype[85-89].

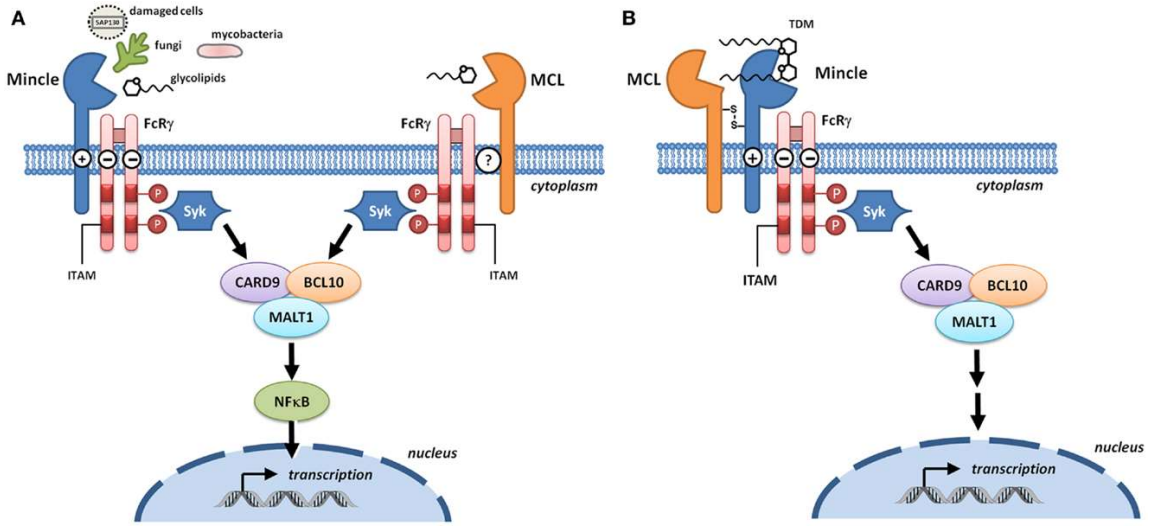

Fig. 11 Signaling through Mincle and MCL. Mark B. Richardson and Spencer J. Williams, "MCL and Mincle: C-type lectin receptors that sense damaged self and pathogen-associated molecular patterns", Frontiers in immunology 2014;5:288[90]

MCL and Mincle recognize a plethora of ligands from fungus to bacteria or endogenous proteins, as summarized in table 1.

Table 1: Overview of microbial and endogenous ligands of Mcl and Mincle

Ligand	Comments	References
Mcl (Clec4d)		
Klebsiella pneumoniae	Protective role in infection model	91
Mycobacterium tuberculosis, *Mycobacterium bovis*	Protective role in infection model	92
TDM from *Mycobacterium* spp.	Mouse, human, not guinea pig	93-96
Blastomyces dermatitidis	Mouse	97
Candida albicans	Controversial role in infection models	98,99
Cryptococcus neoformans	Protective role in infection model	100
Mincle (Clec4e)		
Cholesterol crystals (endogenous)	CRAC motif, human, not mouse/rat	101
SAP130, dead cells (endogenous)	Ca2+-independent, VEGQ motif	102-107
Cholesterol sulfate (endogenous)	Mouse	108
B-glucosylceramid (endogenous)	Mouse	109
Helicobacter pylori	Human	110
K. pneumoniae	Protective role in infection model	111
Streptococcus pneumoniae	Mouse	112
M. tuberculosis, *M. bovis*, *Mycobacterium smegmatis*	Controversial role in infection models	113-117
Cyclopropane–fatty acid α-glucosyl diglyceride from *Lactobacillus plantarum*	Mouse/human	118
β-Gentiobiosyl diaglycerides from *M. tuberculosis* (H37Ra)	Mouse, not human	119
TDM from *Mycobacterium* spp.	Ca2+-dependent, mouse/human/guinea pig/cow	93-95,113,114,120-123
Synthetic trehalose diesters, including TDB, corynomycolates	Mouse/human	93,95,113,120,121,124-128
Synthetic trehalose monoesters	Mouse	129
Glycerol monomycolate (MMG, GroMM)	Human, not mouse	130
Brartemicin	Mimicks glycolipid binding	131
C. albicans	Mouse/human	122,132,133
Cladophialophora carrionii	Human	134
Fonsecaea pedrosoi, Fonsecaea monophora, Fonsecaea compacta	Mouse/human	134-136
Malassezia furfur, glycolipid	Mouse	126,137

Adapted from Jenny Ostrop and Roland Lang, "Collaboration, and Conflict: Signal Integration of Syk-Coupled C-Type Lectin Receptors", Journal of Immunology 2017;198(4):1403-1414[84]

Mincle has been more extensively studied than MCL and it is still unclear how those two receptors relate to each other. It has been postulated that MCL cannot interact directly with FcRγ due to a missing arginine residue in the stalk region[138,139]. While some groups report that MCL signals by forming a heterodimer with Mincle[140], others have found that it can bind to FcRγ in the absence of Mincle using a serine residue at position 38[141,142] (Fig. 11).

Moreover, the mechanisms mediating receptor surface expression are still unclear. While some studies report that MCL ligand binding induces Mincle expression[141], others show that the receptors stabilize each other's surface expression by forming heterodimers[140,143-145].

f) *In vitro, in vivo* and *ex vivo* methods to study the immune system

(i) Forward genetic and congenic strains

The rat model was the leading choice in research until the development of transgenic mice over 25 years ago. Even though the current strategies for transgenic rats are still very limited, research still benefits in using rat models due to the closer physiology between rats and humans, as compared to mice and humans. Moreover, rats are easier to handle, with a larger size allowing optimal surgery and sampling conditions which make working with rats attractive despite the challenges in the reduced number of commercial reagents. The rat genome was published in 2004[146] and our laboratory sequenced the genome of our in-house Dark Agouti (DA) and Piebold-Viral-Glaxo (PVG) strains. This knowledge, along with defined breeding strategies such as F2 crosses and advanced intercross lines (AILs), allowed us to study genetic susceptibility and resistance to neuroinflammation. By backcrossing the offspring of DA and PVG rats to one of the parental strains for more than 5 generations, we were able to generate congenic strains and assessed their phenotypes in response to inflammation. This gave us the tools for a forward genetic approach in order to identify genes responsible for the observed phenotype (as in paper II).

(ii) Cell culture and *in vitro* stimulation

As mentioned before, one of the advantages of the rat models is the larger organ size. This allows for the primary culture of many different types of cells from harvested organs, in a sufficient amount to test several experimental conditions. Undoubtedly, a major drawback is that by lacking the complexity of an integrated system such as the body and tissue, mechanisms investigated using cells obtained *in vitro* do not closely mirror the original processes *in vivo.* Moreover, the artificial manipulation *in vitro* might generate a cell type that does not exactly reflect cells seen *in vivo.* In paper I, we investigate the difference between *in vitro* generated bone marrow-derived DCs with the aim to make a parallel with their possible *in vivo* counterparts.

(iii) T cell culture and differentiation assay

One can generate T cell lines that are specific for a particular antigen from a pool of T cells of different specificity. After immunizing the rats against the specific antigen subcutaneously, the draining lymph nodes will contain a plethora of activated T cells, some being specific for the antigen of interest. Using rounds of *in vitro* stimulation and amplification one can enrich for antigen-specific T cells. These cells can be manipulated during this phase and then be used for *in vivo* or *in vitro* assays. In paper III we used adoptive transfer of myelin basic protein (MBP) specific T cells to assess the effect of vitamin D on CD4+ T cell pathogenicity.

As previously mentioned, naïve Th cells can become activated into different subsets. The cytokine signature of the cells helps determining their subset specificity (Th1 or Th17). The expression of the transcription factor FoxP3 determines if the cells are regulatory (Tregs).

Resting CD4+ T cells, selected for their lack of the marker CD25, can be artificially activated *in vitro* using anti-CD3 (signal 1) and anti-CD28 (signal 2) antibodies. We used this knowledge to test the effect of signal 3 on determining T cell fate in paper I and paper II.

(iv) Flow cytometry

Flow cytometry is one of the greatest tools in immunological research. It is a laser-based technique that enables determination of the level of expression of surface or intracellular markers by cells in suspension, thanks to their interaction with specific fluorescently-labelled antibodies. Moreover, the flow cytometer can discriminate cells according to their size and granularity. This was the technique most used in this thesis in order to assess activation of different subsets of immune cells, or to determine the level of cytokines produced by T cells *ex vivo* or *in vitro* (paper I, II and III). We also used flow cytometry to assess the phagocytosis of fluorescently-labelled beads and pathogens (paper I).

(v) Bone marrow chimeras

In order to separately study the effect of hematopoietic cells from the cells of the rest of the body one can use bone marrow chimeric animals. Similar to the mouse model, the rat model relies on the ablation of the hosts' hematopoietic stem cells using lethal irradiation and their replacement with new hematopoietic cells from freshly isolated donor bone marrow. Unexpectedly, in the rat model, we also observed the replacement of meningeal and perivascular macrophages by cells derived from the donor bone marrow, impeding in this case the assessment of the effect of those cells alone in paper II.

(vi) siRNA knock-down and blocking antibodies

As technology for transgenic animal is limited in rats, we used the small interfering RNA (siRNA) technology that enables the transient suppression of messenger RNA (mRNA). siRNA target the complementary mRNA for degradation via the RNA-Induced Silencing Complex (RISC). This leads to no translation and a reduction of protein expression. We have successfully used siRNA targeting MCL and Mincle *in vitro* and *in vivo* in paper II.

(vii) qPCR and ELISA

Quantitative polymerase chain reaction (qPCR) allows for the quantification of gene transcript expression in tissues or cells. Level of expression is calculated related to stably expressed housekeeping genes (e.g. Actin). The method relies on the quantification of the transcripts, amplified using sequence-specific primers and detected using a fluorescent dye (i.e. Sybr Green) that intercalates itself with the newly generated double-stranded DNA.

Enzyme-linked immunosorbent assay (ELISA) is an antibody-based technique that allows for the detection of proteins in fluids (serum, CSF, cell media). This method was used to quantify cytokine production by activated bone marrow derived dendritic cells (BMDCs) in paper I.

II. Multiple Sclerosis

a) Clinical manifestation

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system affecting mainly young adults in their late twenties or early thirties. Symptoms for MS are very varied as they depend on the spatial localization of the MS plaque (Fig. 12). MS lesions form areas of focal demyelination and immune infiltration. Neuronal damage also occurs in MS ultimately resulting in a loss of brain volume over time.

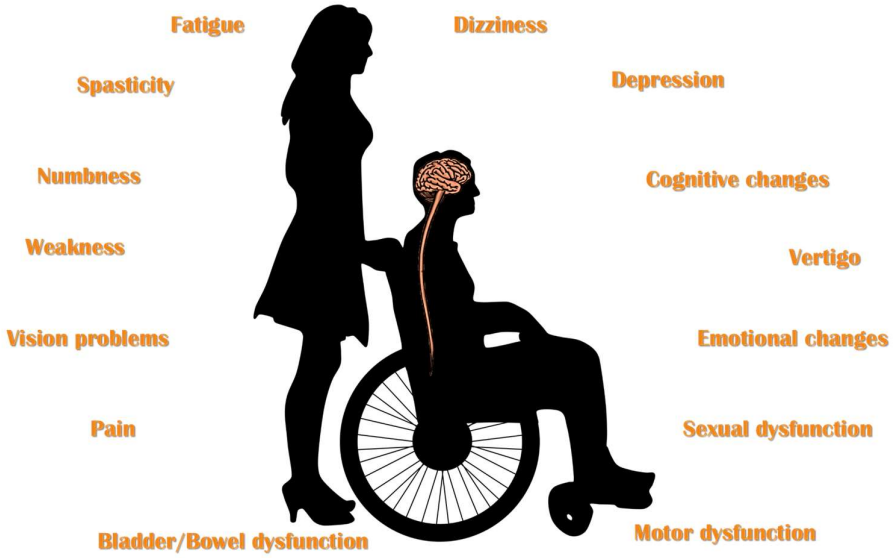

Fig. 12 Symptoms of MS.

MS plaques can be visualized using magnetic resonance imaging (MRI) and their presence is part of the diagnostic criteria for MS[147,148]. There is no straightforward diagnosis for MS as clinicians are still lacking unambiguous test that can alone confirm MS disease specifically. Physicians rely on the inclusion of different diagnostic criteria and the exclusion of other diseases (using blood tests, MRI and CSF analysis) to conclude if a patient is afflicted with MS or not. Most patients accumulate disabilities over time and experience a drastic reduction in their quality of life. It is interesting to note that not all MS lesions will give physical symptoms, suggesting that by the time a patient receives an MS diagnosis they might have already been afflicted by the disease for several years albeit under a sub-clinical threshold[149-151] (Fig. 13).

Clinically isolated syndrome (CIS) could be considered the first clinical form of MS manifestation. It is a first occurrence of neurological symptoms due to inflammation or demyelination in the CNS. CIS patients with MRI lesions have a high risk of converting to MS[152]. The majority of MS patients experience a relapsing remitting pattern in their disease course (RRMS), with bouts of inflammatory attacks followed by periods of remission during which full or partial recovery can occur. Most RRMS patients will enter a second progressive phase of the disease (SPMS) with a gradual increase of disabilities with or without evidence of disease activity by MRI. About 10% of patients present a progressive form of the disease (PPMS), usually at a later age of onset, with steady functional decline without remission[153] (Fig. 13).

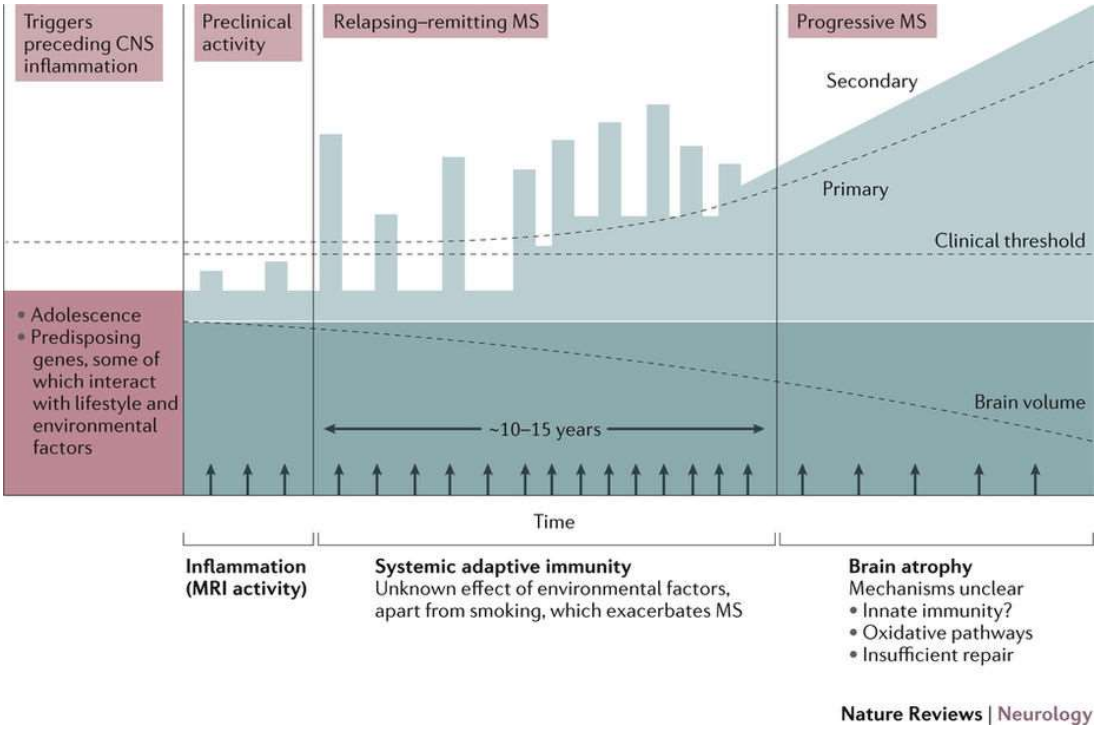

Fig. 13 Evolution of multiple sclerosis. Tomas Olsson, Lisa F. Barcellos and Lars Alfredsson, "Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis", Nature reviews neurology 2016;13:25–36[150]

b) Etiology, risk factors, biomarkers and pathogenesis

(i) Etiology

The cause of MS is still unknown but many genetic risk variants and environmental factors that can contribute to disease have been identified. It is believed that the interaction of both genetic and the environmental risk factors can favor the development of MS. However, different individuals might display different combinations of effects leading to disease. One can therefore question whether MS is a disease with a common pathogenesis or a syndrome due to different mechanisms.

MS lesions can affect all areas of the CNS, including the brain (brainstem, juxtacortical and perivascular white matter, cerebellum), spinal cord (subpial white matter) and optic nerve[154,155]. Lucchinetti and colleagues have described four patterns of early active demyelinating MS lesions from autopsy and biopsy material[156]. Pattern I represents 15% of cases and cells in the lesion consist of macrophages and T cells; pattern II is observed in 56% of patients with the presence of complement and/or antibodies; pattern III is seen in 26% and biopsies show cellular infiltration plus oligodendrocyte death in plaque and peri-plaque; pattern IV is very rare and while it shows very few immune cells there is a profound oligodendropathy in the plaque and peri-plaque. All patterns show the presence of myeloid phagocytes and T cells, with phagocytes outnumbering lymphocytes. While patterns I and II represent plaques in which myelin is destroyed with some preservation of oligodendrocytes and remyelination, pattern II and IV exhibit hyper-sensibility of oligodendrocytes to the inflammatory response resulting in their death and reduced remyelination[155]. However, it

seems that all the active plaques within the same patients are of the same pattern[157,158]. While there is intra-patient homogeneity, the inter-patient heterogeneity supports the possibility that the underlying pathogenesis relies on different mechanisms. It would be interesting to determine whether patients presenting pattern II or IV have a more severe disease progression.

(ii) Risk genes and environmental factors

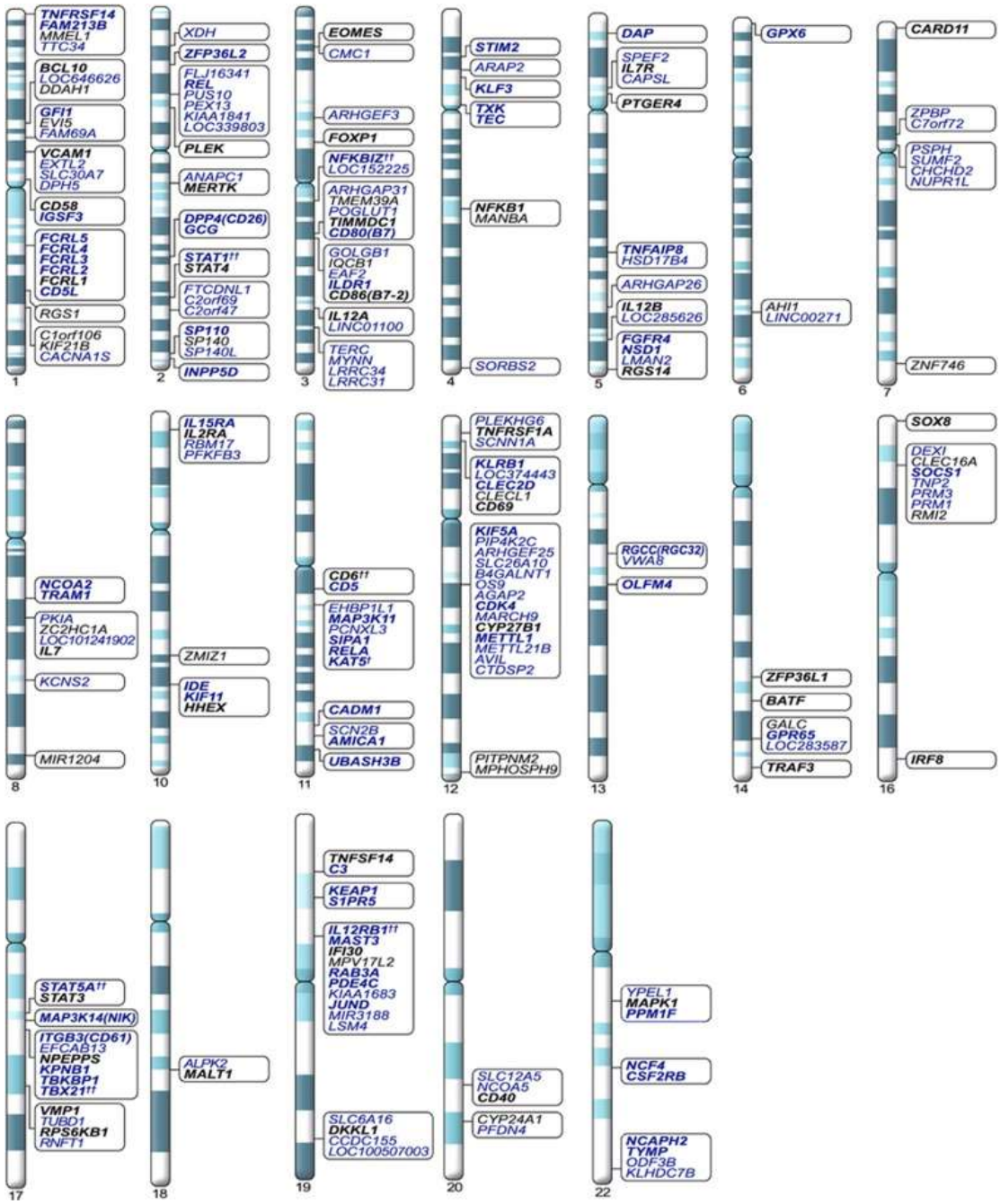

Fig. 14 Significant non-MHC association clusters in MS identified by GWAS-NR. J P Hussman, A H Beecham, M Schmidt, E R Martin, J L McCauley, J M Vance, J L Haines and M A Pericak-Vance, "GWAS analysis implicates NF-κB-mediated induction of inflammatory T cells in multiple sclerosis", Genes and immunity 2016;17(5):305–312[159]

Genome wide association studies (GWAS) have identified over 200 risk variants that can alter the risk for MS[160]. The strongest association maps to the human leukocyte antigen (HLA) class II region (*HLA-DRB1*15:01* haplotype)[161] and most of the other non-HLA risk loci include genes modulating the immune system[162]. This supports the notion that MS is an immune-mediated disease of the CNS rather than a primary CNS degenerative disease. MS is a complex disease and each identified risk locus has small effect on disease risk. Single nucleotide polymorphisms (SNPs) can be located in coding or non-coding regions of the genome. When they occur in coding areas they might change the amino acid sequence (non-synonymous) or not (synonymous), and impact gene expression of the affected gene. When in non-coding regions they might primarily affect gene expression not only of the nearest gene but also distal ones via diverse mechanisms (e.g. enhancer/promoter region)[163]. This makes understanding which genes are affected by a SNP not a trivial matter. Moreover, association studies rely on the genotyping of tag SNPs representative of haplotype blocks (segment of DNA with genes that are often inherited together), which, together with the extensive polymorphic nature of some immune-related loci, e.g. the MHC locus, muddied even more the search for the causative gene(s)[164].

Nonetheless, putative risk genes have been identified and several are involved in signaling by a broad range of adaptive and innate immune cells. Of particular interest to us are BCL10 and MALT1 that participate in T, B and myeloid cell responses via the NF-κB pathway including the response to the CLRs, MCL and Mincle[159,165] (Fig. 14).

The disease discordance between monozygotic twins[166] and the increased incidence and prevalence of autoimmune diseases worldwide[167-171] suggest that in addition to the genetic risk factors, many lifestyle and environmental factors can affect the risk of developing autoimmune states such as MS (Table 2). Some of the strongest risk comes from smoking, lack of sun exposure/ vitamin D deficiency, diet or infections[150,172-179].

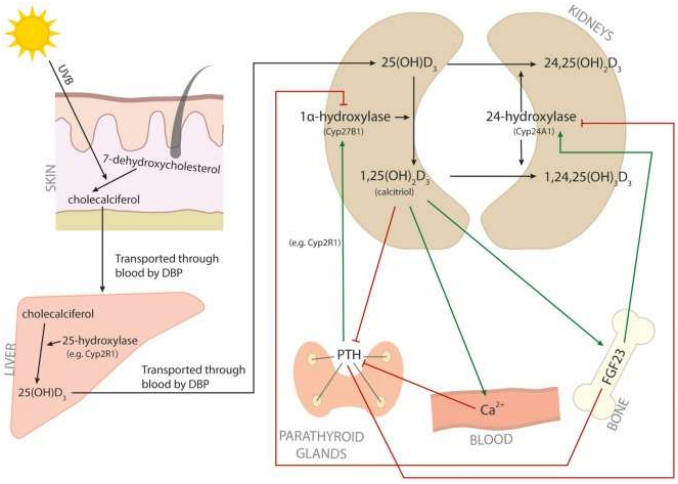

Fig. 15 Vitamin D metabolism. Wendy Dankers, Edgar M. Colin, Jan Piet van Hamburg and Erik Lubberts, "Vitamin D in Autoimmunity: Molecular Mechanisms and Therapeutic Potential", Frontiers in Immunology 2017;7:697

Table 2: Established and possible lifestyle and environmental risk factors for MS

Factor	OR	HLA gene interaction	Combined OR (nongenetic factor + HLA allele)	Effect during adolescence	Immune system implied	Level of evidence
Smoking	~1.6	Yes	14	No	Yes	+++
EBV infection (seropositivity)	~3.6	Yes	~15	Yes	Yes	+++
Vitamin D level <50 nM	~1.4	No	NA	Probably	Yes	+++
Adolescent obesity (BMI >27 at age 20 years)	~2	Yes	~15	Yes	Yes	+++
CMV infection (seropositivity)	0.7	No	NA	Unknown	Yes	++
Night work	~1.7	No	NA	Yes	Yes	++
Low sun exposure	~2	No	NA	Probably	Yes	++
Infectious mononucleosis	~2	Yes	7	Yes	Yes	++
Passive smoking	~1.3	Yes	6	No	Yes	+
Organic solvent exposure	~1.5	Unknown	Unknown	Unknown	Unknown	+
Oral tobacco/nicotine	0.5	No	NA	Unknown	Yes	+
Alcohol	~0.6	No	NA	Unknown	Yes	+
Coffee	~0.7	No	NA	Unknown	Yes	+

Level of evidence for a role of a particular lifestyle or environmental factors in MS is not easy to define. Large prospective studies are, with few exceptions, rare in MS. CMV, cytomegalovirus; EBV, Epstein–Barr virus; HLA, human leukocyte antigen; MS, multiple sclerosis; NA, not applicable; OR, odds ratio; +++, high level of evidence: drawn from large prospective studies or if a case–control observation is supported by Mendelian randomization studies; ++, Case–control observations, if replicated and/or supported by independent methods; +, Non-replicated observations (included to enable further observations).

Adapted from Tomas Olsson, Lisa F. Barcellos and Lars Alfredsson, "Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis", Nature reviews neurology 2016;13:25–36

Vitamin D deficiency may play a role in many autoimmune diseases other than MS, such as SLE, Crohn's disease, type I diabetes and rheumatoid arthritis[180]. While vitamin D (cholecalciferol) can be obtained via the diet, the main source is produced in the skin in response to UV lights (Fig. 15).

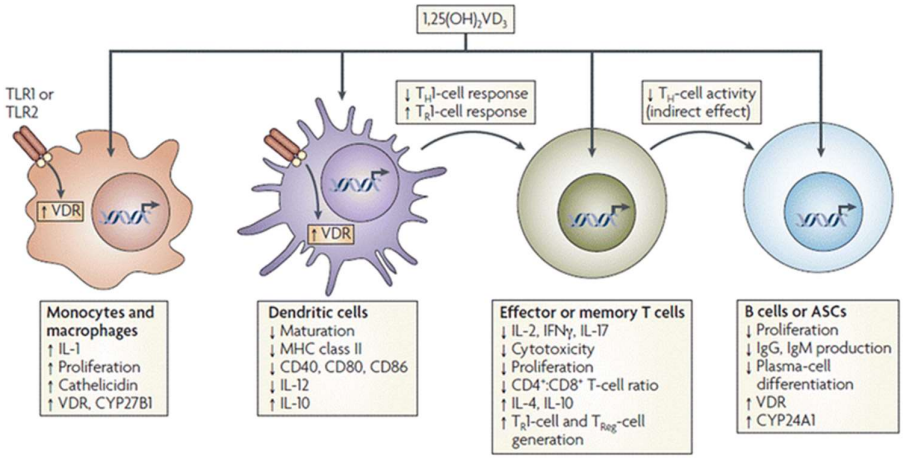

Fig. 16 Potential mechanisms of vitamin D immunomodulation. Martina B. Sintzel, Mark Rametta and Anthony T. Reder, "Vitamin D and Multiple Sclerosis: A Comprehensive Review", Neurology and Therapy 2017:1-27[181]

(iii) Pathogenesis (innate/adaptive/CNS immune system, CNS resident cells, meningeal inflammation)

While most people have autoreactive T cells, not everybody develops MS. The reason for this could be due to two non-mutually exclusive hypotheses: the immune system of MS patients is more reactive, or their CNS is more sensitive to attack due to intrinsic pathologies or infections. The numerous risk loci affecting the immune system and the different patterns of lesions support both possibilities. Moreover, myelin reactive T cells of MS patient produce more pro-inflammatory cytokines (IFNγ, IL-17, GM-CSF) and less anti-inflammatory cytokines (IL-10) than those of controls[182].

The different phases of disease observed in MS patients seem to be due to different sources of immune cells. While the relapsing/remitting phase seems to depend on the entry of peripheral immune cells to the CNS, the progressive phase of the disease seems to be dependent on inflammatory events sequestered in the CNS with little input from peripheral cells[183]. RRMS is thought to be driven by activated self-reactive CD4+ T cells that are present in the CNS and promote the entry of other immune cells, as well as the release of inflammatory mediators [184,185](Fig. 16). This hypothesis is supported by the strong association of the disease with HLA-II molecules necessary to present antigen to CD4+ T cells and by the presence of phagocytic myeloid cells in the lesions. Th1 and Th17 cells are the main effector cells and can promote the activation and recruitment of myeloid cells. Both subsets can activate macrophages and while Th1 cells lead to the recruitment of monocytes, Th17 cells lead to the recruitment of neutrophils. Activated phagocytes, antibodies and complement can cause the destruction of myelin and damage to oligodendrocytes and neurons. The antigen(s) responsible for MS is still unknown. Furthermore, it is still a debate whether the pathological mechanisms of MS develop from a CNS extrinsic or intrinsic event[186]. According to the extrinsic hypothesis, the primary activation of T cells occurs in the periphery due to bystander activation, molecular mimicry or other mechanisms (implying bacterial/viral infection or gut dysbiosis) and reactivation of the T cells ensues as part of the CNS immunosurveillance, leading to the recruitment of more immune cells in the CNS. According to the intrinsic theory, a CNS-specific event (such as viral infection or oligodendropathy) leads to damage and

recruitment of peripheral immune cells (including self-reactive T cells) and inflammation. In any case, infiltration of peripheral immune cells results in the formation of MS lesions[156] where demyelination and subsequent oligodendrocytes and axonal damage can occur. Animal models supporting both hypotheses will be discussed in the section III.

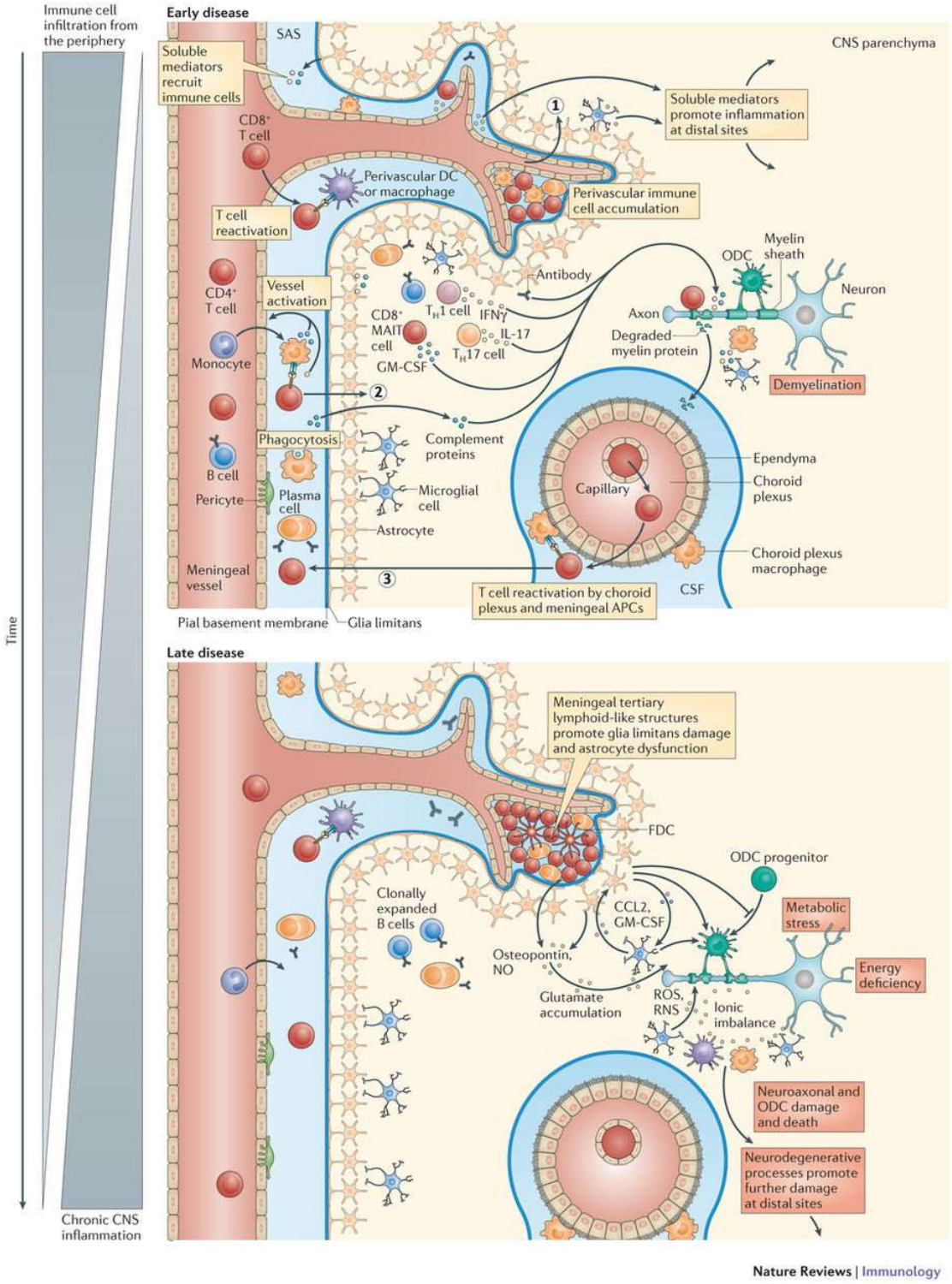

Fig. 17 Immune system dysregulation inside the CNS in early and late multiple sclerosis. Calliope A. Dendrou, Lars Fugger and Manuel A. Friese, "Immunopathology of multiple sclerosis", nature reviews immunology 2015;15:545–558[183]

As the disease progresses the chronic inflammation seems to be restricted to the CNS with little input from peripheral immune cells[71,187] (Fig. 17). In SPMS, immune cells are found in organized tertiary lymphoid structures in the meninges containing T cells, B cells, plasma cells and dendritic cells. A more diffuse cellular infiltration of the meninges is evident in PPMS. Nevertheless, those cells can drive a local chronic inflammation leading to glial dysfunction and cortical damage. The inflammatory products promote metabolic and oxidative stress in neurons, causing neurodegeneration and brain atrophy[188].

Since the most effective therapy to date for RRMS and the only approved therapy for PPMS targets $CD20^+$ B cells[189-192] there has been a renewed interest in their role in MS pathogenesis[70]. B cells are a minor part of the inflammatory infiltrate in active RRMS lesions[156]. However, there seems to be increasing evidence for the accumulation of B cells in the leptomeninges as the disease progresses[183,193,194]. B cells can promote disease pathogenesis by secreting autoantigen-specific antibodies and proinflammatory cytokines, as well as serving as antigen presenting cells and promoting T cell activation.

c) Current and upcoming treatments

When deciding which treatment a MS patient should receive, many factors come into question. Should the patient get a milder but safer therapy in terms of adverse side-effects, or a more potent but risky one? There is still a blatant lack of biomarkers to determine how severe or how fast the disease is going to evolve in patients, which makes the decision even more difficult. Following the initial approval of IFNβ as a MS drug in the 1990s, many new immune modulatory therapies are now available for MS patients, with a diverse range of efficacy and tolerability[195] (Table 3). Most of these treatments are able to delay (reduce relapse rate) but not halt clinical course.

IFNβ along with glatiramer acetate are typically employed as first line therapy. Second line therapies comprise newer drugs that target different aspects of immune functions. The latest approved treatment is monoclonal antibodies targeting $CD20^+$ B lymphocytes (Rituximab, Ocrelizumab, and Oftatumumab) that is currently the most efficient therapy for the treatment of RRMS and also fairly well tolerated[196]. However, this therapy has modest effects on PPMS[197]. Another drastic therapy that is efficient for treating more aggressive forms of MS but is higher in risk is autologous hematopoietic stem cell transplantation (aHSCT)[198,199]. The principal of this therapy is to completely reboot the peripheral immune system using the patient's own bone marrow stem cells. Patients treated using aHSCT have a higher reduction in relapse rate for longer times than with other treatments, even though some patients continue to progress.

The recurrent lack of effect on disease progressions could be due to the confinement of the inflammatory response in the CNS. It seems that meningeal inflammation could be sufficient to maintain the inflammatory response in the CNS independent of further recruitment of immune cells from the periphery. Another possibility is that the chronic inflammatory response in the CNS and CSF leads to irreversible and cascading neuronal damage and loss of function.

Table 3: FDA-approved disease-modifying treatments for MS

Generic (Administration)	Manufacturer (Trade Name)	FDA-Approved Indication	FDA Warnings
Interferon beta-1a (IFNbeta-1a) (Injection: Avonex-weekly, Rebif-thrice weekly. Plegridy – every 2 weeks)	Biogen (Avonex®)	May 17, 1996, for CIS and RRMS	Yes
	EMD Serono (Rebif®)	August 2014 (Plegridy)	Yes
		March 7, 2002, for RRMS	
Interferon beta-1b (IFNbeta-1b) (Injection every other day)	Bayer Healthcare Pharms (Betaseron®)	July 23, 1993, for RRMS	Yes
	Novartis (Extavia®)	August 14, 2009, for CIS and RRMS	Yes
Glatiramer acetate (Injection-daily)	Teva (Copaxone®)	December 20, 1996, for RRMS	Yes
Mitoxantrone (IV)	Bedford; Hospira; Teva Parenteral; Fresenius; EMD Serono Inc., Kabi USA, Mylan Institutional; Onco Therapies LTD	2000 for RRMS, SPMS, PRMS	Yes, black box
Natalizumab (IV)	Biogen (Tysabri®)	November 23, 2004, for RRMS	Yes, black box
Teriflunomide	Sanofi Aventis US (Aubagio) (leflunomide by Sanofi Aventis US as Arava for arthritis)	September 12, 2012, for RRMS	Yes, black box
Fingolimod (Oral)	Novartis (Gilenya)	September 21, 2010, RRMS	Yes
Dimethyl fumarate (Oral)	Biogen (Tecfidera)	March 27, 2013, RRMS	Yes
Alemtuzumab (Injection)	Genzyme (Lemtrada)	November, 2014, RRMS	Yes

CIS = clinically isolated syndrome; FDA = U.S. Food and Drug Administration; IV = intravenous; MS=multiple sclerosis; PRMS=primary relapsing multiple sclerosis; RRMS = relapsing-remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis

Adapted from Butler M, Forte ML, Schwehr N, *et al*, "Decisional Dilemmas in Discontinuing Prolonged Disease-Modifying Treatment for Multiple Sclerosis", Comparative Effectiveness Reviews 2015;150[200]

III. Experimental autoimmune encephalomyelitis as a model of MS

a) Discovery and development of the model

The story of how one of the most used animal models for MS was discovered and developed is fascinating. Rabies is a fatal disease that is caused by infection with *lyssaviruses*. The vaccine for rabies, developed by Louis Pasteur, was made using weakened virion extracted from dried rabbit CNS and it was noticed that some of the vaccinated patients developed encephalitis that was not due to viral infection. Thereafter, the first model of experimental encephalitis was developed in animals using repetitive injection of CNS extract but it had low incidence and, what we would now consider a late disease onset. The discovery that the use of Freund's adjuvant (IFA or CFA) strongly boosted the disease incidence after one or two injections made the model more robust in many species[201]. However, induction of disease in mice was still a problem until it was noticed that it required additional injection of pertussis toxin (PTX)[202,203].

Later, it was found that different CNS proteins could be used to induce disease with different clinical courses (monophasic, relapsing remitting, chronic) in different strains and that some strains were more susceptible and other more resistant to disease induction[184]. It is interesting to note that there are similarities but also differences in the lesion patterns between MS (RRMS and SPMS), human encephalitis due to rabies vaccine, and the EAE models in different rat strains (Fig. 18). In all cases primary demyelinating regions with immune cell infiltration are evident but while the plaques could affect either brain or spinal cord in MS or post rabies vaccination, they are mainly found in the spinal cord in the EAE model.

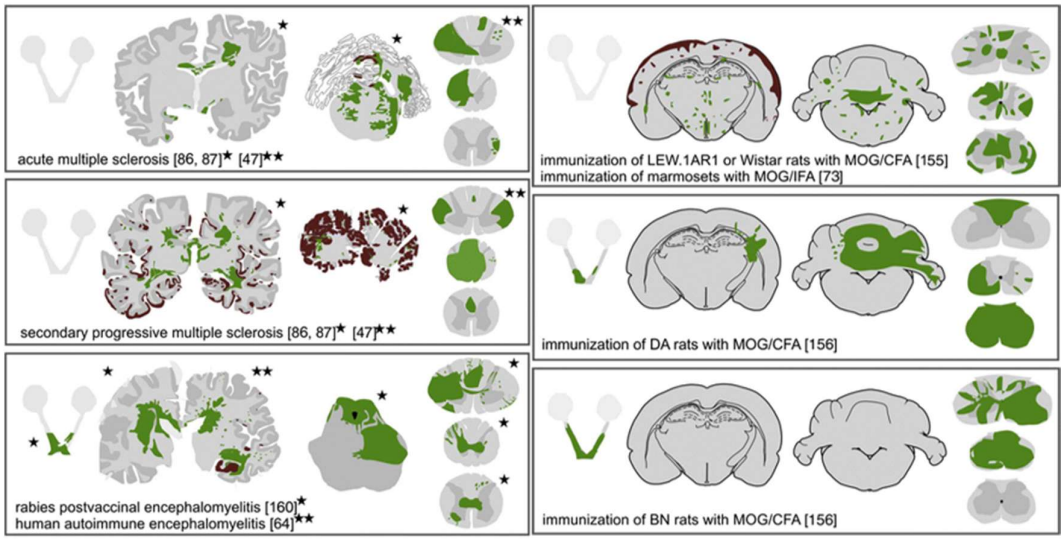

Fig. 18 Distribution of demyelinating lesions in MS and different EAE-based models. Hans Lassmann and Monika Bradl, "Multiple sclerosis: experimental models and reality", Acta neuropathologica 2017;133(2):223–244[184]

EAE: active, passive and spontaneous models

The work of this thesis is based on the active EAE model in the rat using immunization with recombinant myelin oligodendrocyte glycoprotein (MOG) emulsified in PBS/oil (incomplete Freund's adjuvant, IFA). Under this immunization protocol the DA rats are susceptible to disease but the PVG rats are resistant. Our group has spent numerous years to identify genomic regions affecting susceptibility or resistance to EAE and to determine the candidate genes and possible mechanisms involved. DA rats present with a relapsing-remitting disease, possibly due to epitope spreading[204]. Rats are afflicted by an ascending paralysis visually assessed using a scoring system of 1-5: score 1 limp tail, score 2 hind limb weakness/affected gait, score 3 hind limbs paralysis, score 4 front limb paralysis and score 5 moribund state. Lesions most likely start at the lumbar region of the spinal cord and propagate along it, and demyelination can also be observed in the hindbrain[205].

We have also used passive EAE that is achieved by transferring myelin-specific T cell lines established *in vitro* using MBP to recipient animals intravenously. This model is mainly used to study the effect of T cells in EAE and yields a monophasic disease course with no demyelination. Transfer of MBP-specific T cells along with pathogenic autoantibodies specific for myelin can induce demyelination. Our lab also acquired a green fluorescent protein (GFP)

transgenic DA rat strain (DA-GFP) that allowed us to track GFP MBP-T cell lines injected into non-GFP recipients or *vice versa*.

The mouse model of EAE is now more frequently used than the rat model, mainly due to the availability of many different transgenic mouse strains. Several strains of mice were developed in which the T cells are all myelin antigen-specific[206,207], the most used being the 2D2 mouse strain on the C57BL/6 background. These mice develop spontaneous EAE with a low incidence (less than 5%), but if these mice are crossed with another strain where B cells are also specific for MOG then the incidence of spontaneous EAE increases drastically (50% and more)[208]. There is currently no spontaneous rat model of MS.

b) Other animal models of MS

It is important to note that while the EAE model has been very useful in understanding some pathogenic mechanisms underlying MS, this model only reflects some but not all aspects of the human disease. Furthermore, while we definitely know the antigen in EAE, the trigger(s) for MS still remains unknown and the strong artificial induction of the immune response in EAE (especially with CFA and/or PTX) biases the immune system in ways that may or may not reflect the immune response in MS. There are other models that also recapitulate histopathological features of MS, among them being the viral-induced model and a novel interesting model of neuroinflammation following primary oligodendropathy. Most of these models have been developed in mice.

Viruses have long been considered potential triggers of MS. While not one specific virus has been linked to disease there are several candidates, such as Epstein-Barr virus or cytomegalovirus. The viral-induced animal models support this hypothesis and show that an acute pan-encephalitic phase is followed by a second phase in which the immune system causes chronic inflammation and demyelination. The two main models are caused by Theiler's Murine Encephalomyelitis Virus and the mouse hepatitis virus (MHV) strain JHM, respectively[209,210]. Inflammatory infiltrates consist of lymphocytes and demyelinating activated macrophages/microglia.

The possibility that the autoimmune response observed in MS could be due to a primary oligodendropathy has always been a debate. Even though some types of lesions exhibit aspects of oligodendrocyte pathology, it is difficult to determine if the death of oligodendrocytes is a cause or a consequence of the ongoing inflammation. This is particularly true since by the time patients are diagnosed with MS they have most likely already been afflicted by the disease for many years. In 2009, Weissman and colleagues reported a study in which they attempted to trigger autoimmunity following oligodendrocyte death[211]. In this model, mice expressing the diphtheria toxin receptor on oligodendrocytes were injected with the toxin that induces oligodendrocyte death and myelin damage. Despite seeing the accumulation of myelin components in the draining lymph nodes they did not observe the development of CNS autoimmunity, even when they boosted the immune system by different methods such as Treg ablation or activation of APCs with anti-CD40 antibodies. It is important to note that following diphtheria toxin injection these mice exhibited a severe phenotype and died prematurely. However, in 2015, Popko and colleagues reported induction of immune-

mediated CNS demyelination following oligodendrocyte death[212]. The model was slightly different and used the expression of diphtheria toxin directly in oligodendrocytes, induced by tamoxifen which resulted in widespread demyelination. However, the animals survived and developed a secondary neuroinflammation and demyelination almost 10 months later with increased numbers of $CD4^+$ T cells and myeloid cells in the CNS compared to controls. These findings support the 'inside-out' hypothesis that a CNS intrinsic event can cause autoimmunity in MS.

c) EAE pathogenesis

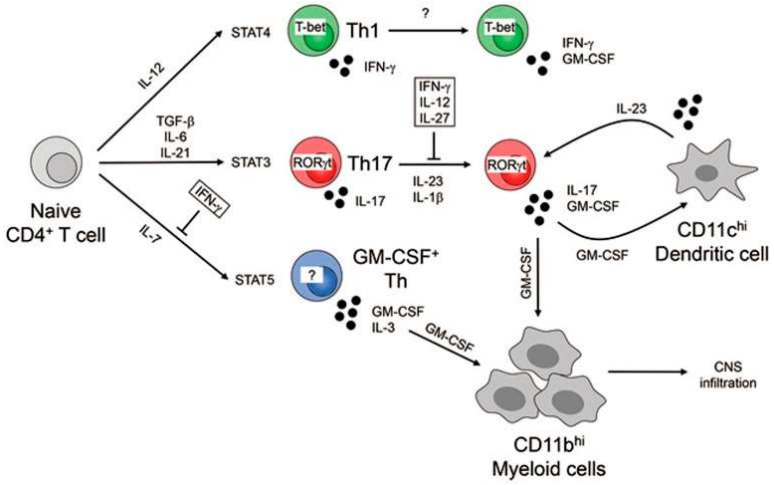

Fig. 19 The role of T helper cell subsets and the cytokine environment in driving autoimmune inflammation. Dietmar Herndler-Brandstetter and Richard A Flavell, "Producing GM-CSF: a unique T helper subset?", Cell Research 2014;24(12):1379–1380[213]

The EAE model relies on the pathogenic action of $CD4^+$ T cells and how they orchestrate a subsequent inflammatory response in the CNS leading to demyelination, oligodendrocyte death and axonal damage[214]. My thesis focuses on the recombinant MOG-EAE model in the DA rat, but most of the current knowledge of EAE arises on studies of mice. The DA-MOG model depends not only on the generation of MOG-specific T cells but also the presence of MOG-specific demyelinating antibodies[204]. The different immunization protocols in EAE boost the generation of antigen-specific CD4 T helper cells (Fig. 19). For a long time, Th1 $CD4^+$ cells were considered to be the pathogenic T cell subset responsible for disease. Th1 cells are generated via the transcription factor Tbet after their activation in the presence of IFNγ and IL-12 (signal 3), as mice deficient for the subunit of IL-12, IL-12p40, were resistant to EAE[215]. However mice deficient in the IL-12p35 subunit were not protected and developed exacerbated disease[215], and this was also true for IFNγ-deficient mice[216]. It was then understood that the IL-12p40 subunit was shared between 2 different cytokines, IL-12 and IL-23, and that IL-23p19-deficient mice were also resistant to EAE[217,218]. This led to the hypothesis that another subset of T helper cells might be pathogenic, namely Th17 which are generated via the transcription factor RORγt in the presence of the cytokines IL-1β, IL-6 and TGFβ. While IL-23 is necessary for Th17 cells to become pathogenic by promoting their production of GM-CSF, IL-1β promotes GM-CSF production[219,220]. GM-CSF in turn can induce IL-23 production by APCs. Unsurprisingly, mice deficient in RORγt, IL-6, IL-1β or GM-CSF are all resistant to EAE[221-

[223]. However, mice deficient in IL-17a, the hallmark cytokine of Th17 cells, are partially or not resistant[224,225]. These findings seem to pinpoint the importance of GM-CSF production by Th cells for EAE pathogenesis and a new subset of T cells called GM-CSF$^+$ Th cells generated with IL-2 or IL-7 and dependent on the transcription factor STAT5[226,227]. While most of the work was done on Th17, Th1 cells, even in the absence of RORγt, can produced robust amounts of GM-CSF through other unknown regulatory mechanisms.

All three subsets of T helper cells, Th1, Th17 and GM-CSF$^+$ Th, can induce passive EAE when transferred into recipient animals. Furthermore, Th differentiation seems to be highly plastic and influenced by the cytokine milieu. In the CNS of EAE affected mice, fate mapping experiments have shown that there are many ex-IL17 producing that have switched to IFNγ production[228] (Fig. 7). It is very likely that all subsets might have a role at one point in time or another in MS pathogenesis as many Th cytokines levels are increased in MS patients (Table 4).

Table 4 : Major cytokines contributing to the pathogenesis of MS and EAE

Cytokine	Main Producers	Levels in MS Patients	Role in EAE	Potential Treatments of MS
GM-CSF	T cells	Elevated	GM-CSF-deficient mice are completely resistant to EAE[229]	Phase 1b trial of humanized anti-GM-CSF mAb MOR103 in MS is completed[230]
IFN-β	pDCs	Not reported	Ifnb−/− mice exhibit increased EAE severity[231]	First line treatment of RRMS[232]
IFN-γ	Th1 cells, NK cells, NKT cells	Elevated	Ifng−/− mice exhibit increased EAE severity[216]	Intravenous infusion of IFN-γ exacerbates disease in MS patients[233]
IL-1β	Monocytes, macrophages	Elevated	Il1r1−/− mice are resistant to EAE[234]	Not reported
IL-10	Tregs, macrophages, DCs, B cells	Reduced	Il10−/− mice exhibit increased EAE severity[235]	Not reported
IL-12	DCs, macrophages	Elevated	IL-12 p35−/− exhibit increased EAE severity[236]	Anti-IL-12/IL-23 p40 mAb Ustekinumab does not show efficacy in treating RRMS in phase II trial[237]
IL-17	Th17 cells, γδ T cells, NKT cells	Elevated	Il17a−/− mice are partially resistant to EAE[225]	Anti-17A mAb Secukinumab reduces disease severity in RRMS patients[238]
IL-23	DCs, macrophages	Elevated	Il23r−/− mice are completely resistant to EAE[217]	Anti-IL-12/IL-23 p40 mAb Ustekinumab does not show efficacy in treating RRMS in phase II trial[237]
TNF-α	Macrophages	Elevated	Tnfrsf1a−/− mice are partially resistant to EAE[239]	Treatment of MS patients with anti-TNF-α exacerbates disease in MS patients[240]

GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN: interferon; IL: interleukin; TNF: tumor necrosis factor; DCs: dendritic cells; pDCs: plasmacytoid dendritic cells; NK: natural killer; NKT: nature killer T; Tregs: regulatory T cells; RRMS: relapsing-remitting multiple sclerosis; mAb: monoclonal antibody.

Adapted from Pushpalatha Palle, Kelly L. Monaghan, Sarah M. Milne and Edwin C.K. Wan, "Cytokine Signaling in Multiple Sclerosis and Its Therapeutic Applications". Medical Sciences 2017;5(4):23

Myeloid cells present in the CNS of EAE animals contribute to demyelination and inflammation. However, while CNS parenchymal resident microglia seem to be important for debris clearance and healing, monocytes/macrophages seem to promote demyelination[241,242].

2. Thesis aim

This thesis addressed mechanisms by which genetic (CLRs) and environmental (vitamin D) factors regulate susceptibility to rat EAE induced by immunization with recombinant MOG.

The aim was to study the regulation of pathogenic T cell development in EAE. Furthermore, we assessed the relevance of our findings in the human disease MS, bringing novel insights to the pathological mechanisms underlying disease.

In paper I we provided new light on the functional characteristics of BMDCs frequently used *in vitro* to study the responses of different subsets of myeloid cells. In papers II and III we described two distinct mechanisms affecting the differentiation of pathogenic Th17 and regulating EAE.

3. Results, discussion and future perspectives

The approaches used during my PhD work are part of the strategies employed in our laboratory. The aim of the Neuroimmunology Unit is to study neuroinflammation, particularly in the context of MS, exploring different aspects of disease, from genetic and environmental influences to molecular and cellular mechanisms driving pathogenesis. Our research also relies on a translational approach combining pre-clinical investigation in rodent models of MS and clinical data from human studies, towards the ultimate goal of a better care of MS patients.

In that context, the first paper addresses a basic immunological question: to what extent an *ex-vivo* experimental protocol affects the cellular phenotype of cultured cells, in light with their *in-vivo* counterparts. The "BMDC" project arose from the interest in utilizing bone-marrow derived DCs for a project I started with when I arrived in the Unit. While the initial study did not yield any promising results and was eventually stopped, the experimental setting I performed to generate BMDCs further questioned me on the phenotypic heterogeneity of cultured cells ex-vivo and their potential application for pre-clinical and clinical investigations. In other words, how different are cells in my Petri dish when I use different protocols to generate what is often grouped under the umbrella of 'BMDCs'? What functions can those cells fulfill and what would be the closest *in vivo* counterpart to those *in vitro* generated cells? Our results were in accordance to that reported in mice[243] and we could further complement the findings with a more functional approach.

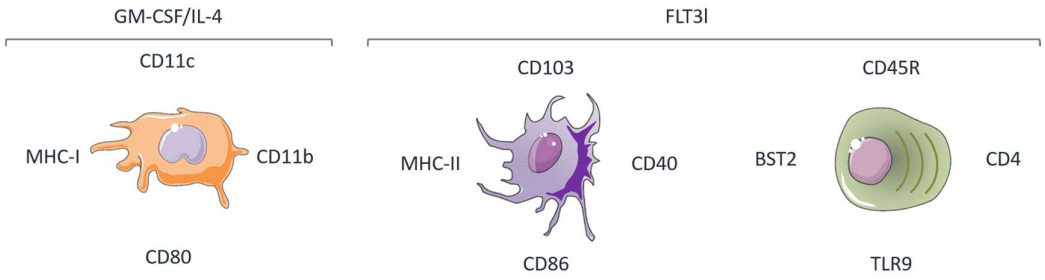

Fig. 20 BMDCs generated with GM-CSF/IL-4 or FLT3l are phenotypically different

Together with my colleague Andreas Warnecke, we demonstrated that, on the one hand, FLT3l-generated BMDCs (FL-BMDCs) were functionally closer to DCs *in vivo*. FL-BMDCs were 2 subpopulations (CD103+ and CD45R+), the CD103+ cells constitutively expressed CD86, CD40 as well as high levels of MHC II (Fig. 20). FL-BMDCs secreted IL-6 and IL-12 and were excellent at stimulating $CD4^+$ T cells, notably towards the Th17 phenotype. Moreover they expressed genes known to be specific for *in vivo* DCs. Conversely, GM-CSF- and IL-4-generated cells (G4-BMDCs) expressed higher levels of MHC-I, CD11b, CD11c as well as CD80 (Fig. 20) and could upregulate MHC II and CD86 upon stimulation. They were excellent producers of pro-inflammatory cytokines (e.g. TNF) and reactive nitrogen species (NO), and favored the differentiation of CD4+ T cells to Tregs. Our work, along with that of others, supports the idea that while FL-BMDCs are phenotypically closer to cDCs, G4-BMDCs are closer to inflammatory monocytes derived cells (MCs), often called DCs for their expression of CD11c.

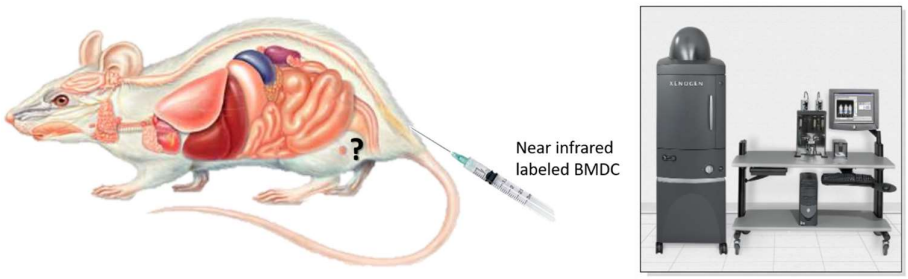

Fig. 21 Schematic for the *in vivo* trafficking experiment using an IVIS machine

An interesting follow-up to this project would be to study the behavior of these cells *in vivo*. We performed preliminary work tracking the migration of subcutaneously injected FL- and G4-BMDCs using the *in vivo* imaging system (IVIS) (Fig. 21) and could detect a preferential migration of FL-BMDCs to the draining LNs while G4-BMDCs primarily remained at the injection site (Fig. 22).

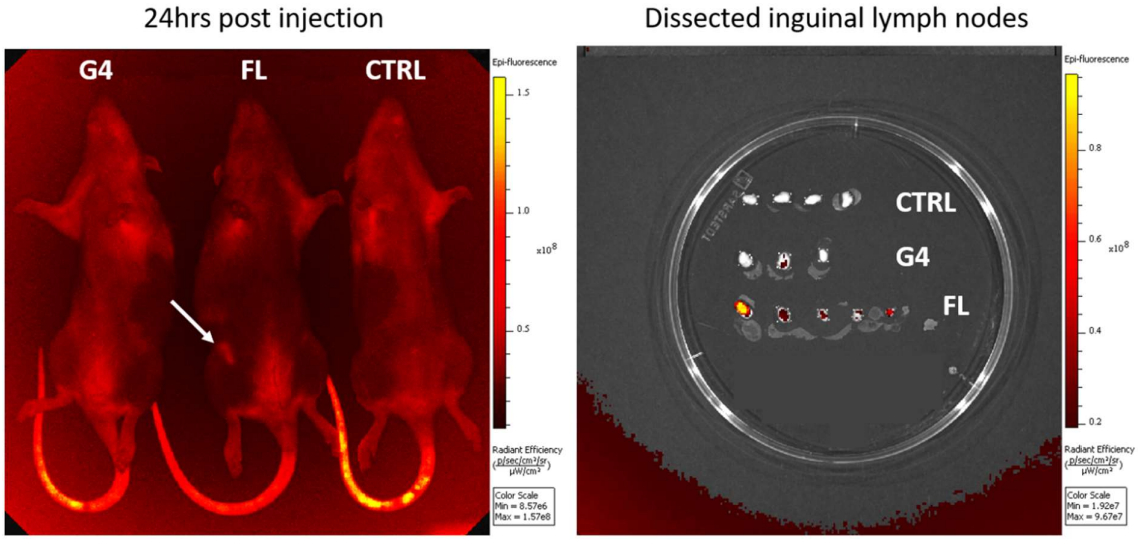

Fig. 22 Tracking of near IR labeled FL- and G4-BMDCs

We also became interested in testing the capacity of these cells to promote immune responses to antigen *in vivo* and came across major challenges in this effort, mainly due to their very high capacity at picking up and presenting antigens from proteins present in the culture media. Time did not permit to continue with this part of the project but there is definitely an interest in using different types of *in vitro* generated myeloid cells for different tasks *in vivo*. Future work could, on one hand, explore the potential of FL-BMDCs in vaccination. As those cells are excellent at activating T cells and promoting Th1 and Th17 cells they would be suitable candidate to initiate an immune response. On the other hand, G4-BMDCs could be investigated in the context of cancer immunology and breaking tolerance. Those cells could play a role in promoting inflammation and should have the capacity to cross present antigen to cytotoxic CD8+ T cells since they express high level of MHC-I.

Our laboratory is interested in neuroinflammation, particularly in the context of MS, and has spent numerous years identifying genetic loci regulating MS (as part of global GWAS studies[244-246]) and EAE (quantitative trait loci identified in rats[247-250]). I joined the group at a time when the focus changed from identifying the genomic regions involved in EAE regulation to narrowing down the gene(s) responsible for the phenotype observed and determining their mechanisms of action. This was the aim of my second paper, which is still in a manuscript form although near completion and has been my main project in the laboratory. I was fortunate to join my colleague Sevasti Flytzani on a project with a very strong phenotype (drastic reduction in EAE incidence in the congenic rats) and a rather small genomic region affecting the phenotype. The evident phenotype promised optimal conditions to "easily" decipher the underlying mechanisms. My naive optimism was quickly mitigated over the years and I appreciated studying how PRRs and DAMPs can contribute to autoimmunity. Our findings so far showed how 2 PRRs of the C-type lectin family, MCL and Mincle, could regulate EAE incidence by affecting the reactivation of $CD4^+$ T cells in the CNS (Fig. 22). The congenic rats (CLRc) had a reduced expression of both receptors compared to DA littermate controls and were protected from EAE. MCL and Mincle are both expressed by myeloid cells and we could detect them on neutrophils, monocytes and macrophages, but not microglia. We observed a defect in T cell activation in the CNS of congenic animals that particularly affected Th17 cells (IL-17a and GM-CSF production and proliferation). This resulted in a reduced recruitment of granulocytes into the CNS. We pinpointed the potential *in vivo* ligand of these two receptors as SF3B3 (SAP130), an endogenous nuclear protein that can be released by necrotic cells. We showed that blocking this protein *in vivo* in the CNS could protect rats from developing EAE. We also started to explore another potential source of SAP130 and speculated that activated macrophages could actively release this protein in a similar manner than the release of HMGB1 by LPS stimulated macrophages[251]. So far, we could discern that macrophages stimulated with TNF could translocate the protein from the nucleus to the cytoplasm but those are still preliminary results. Finally, we could observe hyperactivity in the MCL/Mincle signaling pathway in monocytes from MS patients which could be due to the increased expression of MCL, Mincle and CARD9 during inflammation in MS patients compared to controls.

Our manuscript is the first to demonstrate mechanisms by which CLRs MCL and Mincle could regulate susceptibility to EAE. Indeed, one other previous report has examined the effect of those receptors in EAE in mice[96], but they did not address the mechanisms. Additionally, the authors used the MCL/Mincle ligand TDM as adjuvant in replacement of heat-killed mycobacteria for EAE induction. It is therefore impossible to determine if under those conditions the role of these receptors was important in either the peripheral and/or CNS activation of T cells. Others groups have shown an important role of Mincle in neuroinflammation, such as in experimental autoimmune uveoretinitis (EAU)[252], stroke[104,107,253,254] and traumatic brain injury (TBI)[106]. Interestingly, in EAU the absence of CARD9 and Mincle were linked to a defect in Th17 response[252], whereas in TBI the inflammatory response was associated to Mincle and SAP130[106]. We have shown that the expression of MCL and Mincle in the rat CNS, most likely in myeloid cells of the meninges, was crucial for the reactivation of CD4+ T cells to the Th17 phenotype, and that this effect was

partially due to SAP130. Following the hypothesis that MS could be due to a hyperactive immune system, we could observe an increased response to the MCL/Mince ligand TDB in MS monocytes compared to in healthy controls. We did not observe this difference following stimulation with the TLR4 agonist LPS. This pinpoints the specific dysregulated response of the MCL/Mincle signaling pathway, which could contribute to the overall inflammatory response in MS.

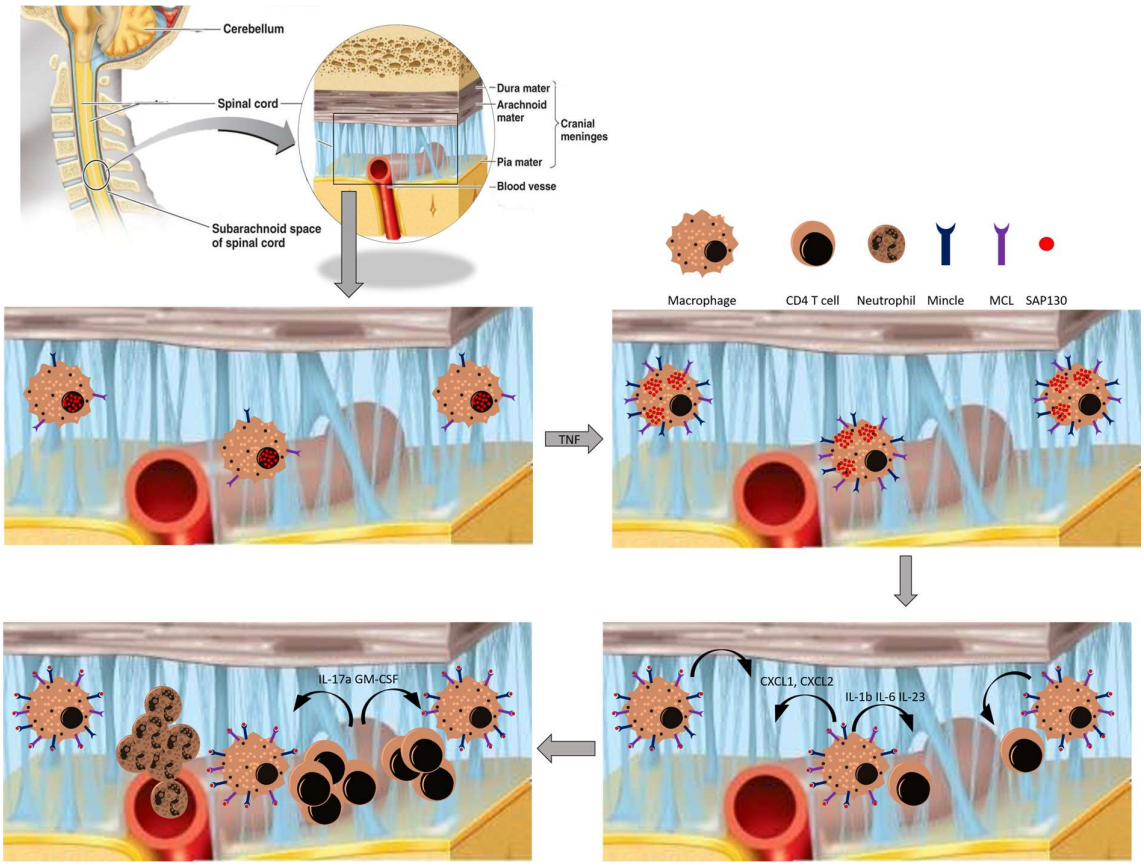

Fig. 23 Possible mechanism of action in the meninges of DA rats which is defective in CLRc congenics due to low expression of MCL and Mincle.

We are still investigating the mechanism of action of SAP130 *in vivo*, particularly since we failed to stimulate macrophages *in vitro* with the commercially available recombinant SAP130 proteins. This could be due to the fact that most of those are partial recombinant proteins and this could affect their capacity to bind to MCL and Mincle. We are attempting to determine if macrophages can release and respond to endogenous SAP130. Moreover, further work is still needed to address the relevance of this rat study for MS pathogenesis and its potential treatment application. It would be very interesting to determine how much of this mechanism observed in rats translates into humans. The study by de Riverro Vaccari *et al* pointed to a role of at least Mincle in neuronal response to SAP130 during TBI suggesting that it could induce neuronal death[106]. One could speculate the occurrence of similar mechanisms in the brain of MS patient as well. To address this question, one could determine MCL and Mincle expression by neurons and test for the accumulation of SAP130 protein in situ by immunohistochemistry in post-mortem brain sections from MS patients compared to non-neurological controls. The

aforementioned diversity of MS lesions further requires careful selection of clinical material. This, together with detection of SAP130 in CSF of MS patients could provide insights into the relevance of SAP130 and MCL/Mincle-related pathways in MS. Importantly, considering the extensive clinical heterogeneity of patients affected with MS, e.g. in disease course, symptoms and severity, one could hypothesize that SAP130/MCL/Mincle-related processes would play a role in a subset of MS patients, affecting neuroinflammatory and possibly neurodegenerative processes. Moreover, more effort is needed in order to decipher the mechanism underlying the observed hyperactive MCL/MINCLE response in MS patients, for example with regards to genetic variation predisposing to such response. Indeed, while considerable effort has been made in identifying genetic risk variants predisposing for disease, much less is known about the genetic factor predisposing for specific cellular response in a context-specific manner. Ultimately, the question whether MCL/MINCLE signaling pathway could be a target for therapy would need to be addressed, our data seems to be pointing at a role of this pathway at the initiation phase of the disease which is not possible to treat in MS as most patients are diagnosed with the disease years after onset. However MCL/MINCLE signaling could still play a role during relapse and therefore be targeted with blocking antibodies for example.

Other than genetics, our laboratory also has a particular interest in environmental factors that affect MS and EAE. Paper III is a follow-up of a previous study[255] that showed the importance of vitamin D in adolescent rats for EAE protection. Interestingly, while the beneficial role of vitamin D in MS is well established, the mechanisms underpinning protection, and more specifically immunomodulation, exerted by vitamin D, are poorly known.

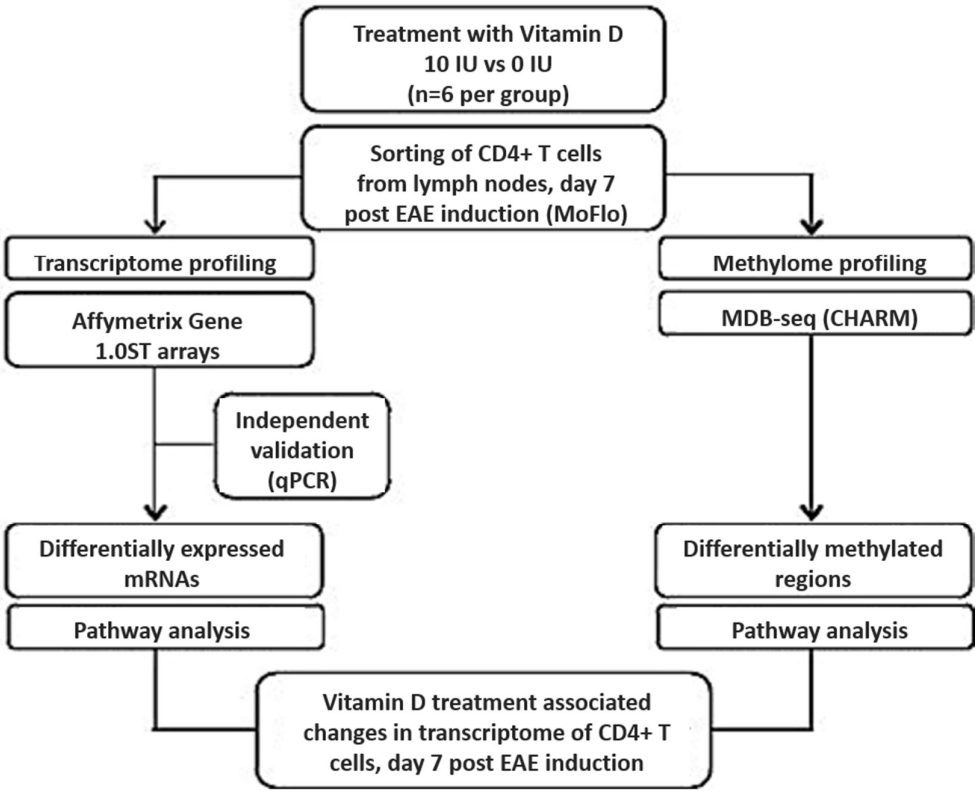

Fig. 24 Schematic illustration of study design from paper III[256]

Herein, using omics approaches in rodents, we described the epigenetic mechanisms of action of vitamin D on CD4+ T cells that affect proliferation and Th17 differentiation. Even though my main supervisor is an expert in epigenetics I personally didn't work directly on epigenetic mechanisms regulating inflammation. My main contribution to this project was from an immunological perspective. When I joined the lab my first project was to establish the passive EAE model with MBP-specific T cells and we used this method to address the effect of vitamin D on T cell activation. The whole paper showed how vitamin D affects CD4+ T cell activation *in vivo* via changes in DNA methylation and miRNA expression, which in turns affected the expression of many genes involved in cytokine and T cell receptor signaling as well as cytokine production (notably IL-2). This led to impaired Th differentiation by the T cells, notably the Th17 phenotype, as well as defect in cell proliferation. We used the adoptive transfer model to prove that even short-term treatment with vitamin D impacted effector T cell activation into Th1, but even more so for Th17 cells and drastically reduced their proliferation. These cells were less encephalitogenic when transferred to the recipient rats compared to untreated cells. This study not only provides a mechanism underlying the effect of vitamin D treatment of juvenile rats on $CD4^+$ T cells and susceptibility to EAE, but also provides functional evidence that vitamin D affects the pathogenic potential of $CD4^+$ T cells directly.

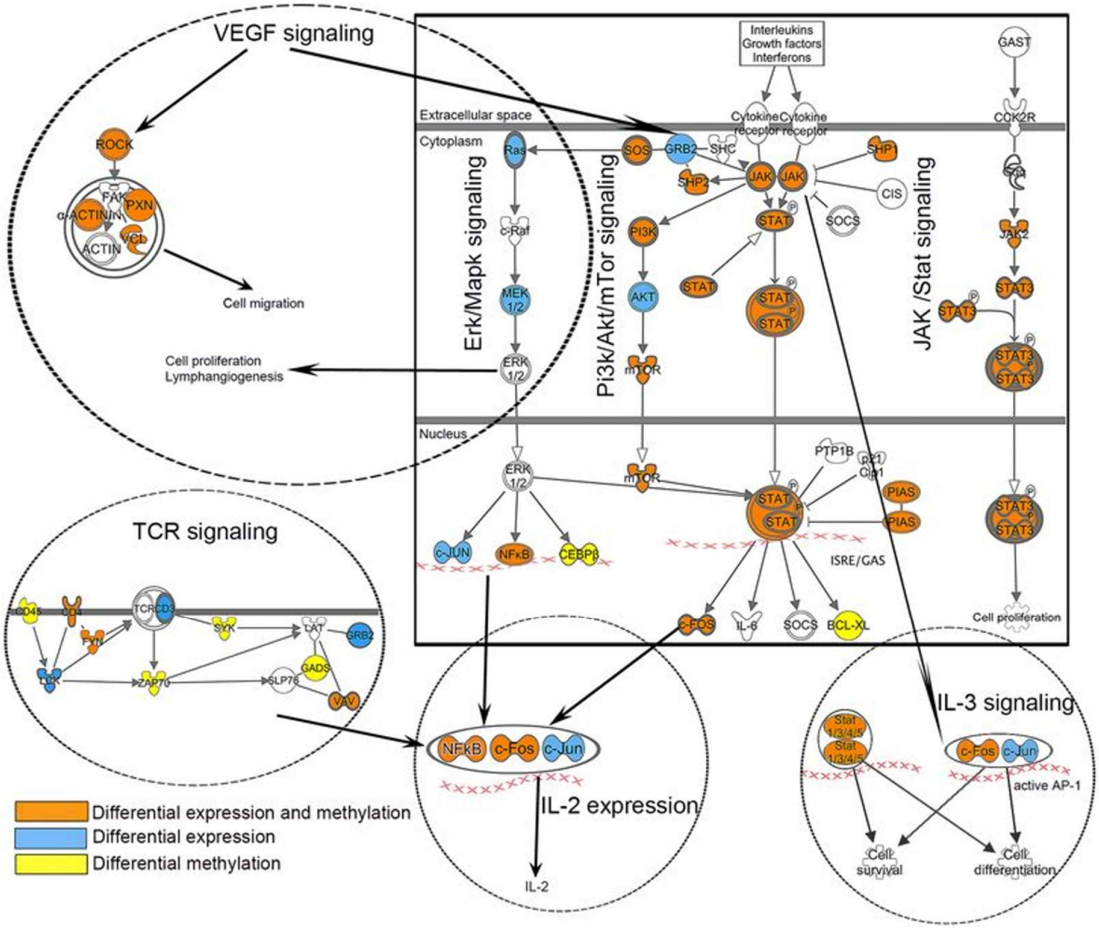

Fig. 25 Schematic representation of Jak/Stat, Erk/Mapk, Pi3k/Akt, Vegf, TCR, and IL-3 signaling that are affected by vitamin D. Orange indicates genes that are differentially expressed and methylated. Blue indicates genes that are differentially expressed only, and yellow indicates genes that are differentially methylated only.[256]

Future work could study the mechanism of vitamin D effect on APCs in a similar manner, as they also express VDR. Also, it would be interesting to distinguish the contribution of vitamin D on the direct response by T cells or the modulation of T cell response via APCs. Our findings, together with other studies, further encourage the use of vitamin D supplementation, probably in case of deficiency during critical timeframe of risk to autoimmunity. In addition to prophylactic perspective, the potential of vitamin D supplementation as a therapeutic tool in patients with established MS disease is still controversial[257]. Examining the effect of vitamin D treatment on PBMC activation *in vitro* in response to antigen (myelin or others), could shed some light on its mechanism of action in humans. In light with our findings, one could speculate about a putative beneficial effect of vitamin D treatment in combination with other conventional immunomodulatory agents.

Overall, using different approaches in rodent models and human material, the work presented here provides additional insight into the immunological aspects of MS-like disease and further highlight the importance of utilizing adequate experimental settings and complementary and integrative strategies to tackle a biological question. Our results support the "hyperactive immune system" theory, showing how PRRs and environmental factors such as vitamin D levels can affect the immune system to promote pathogenic inflammation. There are many more identified risk factors for MS but we are still not nearer to finding a cure, even though the disease modifying therapies have evolved very fast this past decades and are getting more and more efficient at reducing relapse rate and slowing down the disease. More efforts needs to be put on several aspect of MS. There is still many other genes that can contribute to disease and their underlying mechanisms remain unknown. Identifying more functional pathways involved in MS pathogenesis will help to determine how to best treat patients with more targeted therapies. Currently we lack the possibility of knowing whether a patient diagnosed with MS will have a slow disease progression or a more aggressive one. We need better biomarkers to stratify patients into potential mechanistic subgroups that will enable more personalized therapy. Finally, we need a better understanding of CNS intrinsic events that could provide a clue to the disease etiology but also mechanisms of progression. The main limitation of the EAE model is that it is made to boost a CD4+ T cell mediated disease, studying other models such as the viral models or models based on primary neuropathy will help better understand the "more sensitive CNS" theory of MS pathogenesis. It doesn't mean that the EAE model is obsolete but research would benefit from using different models reflecting different aspects of MS. In that context, a bi-directional approach of research combining studies in both human and animal material appears critical. Indeed, determining the relevance of mechanisms identified in animal models using clinical material and, addressing the pathogenic mechanisms of risk factors observed in human using *in vivo/in vitro* laboratory experiments will help understand the disease pathogenesis. Moreover, identifying risk factors affecting disease progression and their underlying mechanism will provide more light on what makes the CNS tip towards degeneration. Furthermore, mechanisms promoting repair in the CNS are crucial and would provide a useful complementary therapy to the current immunomodulatory drugs by promoting recovery. It is no trivial matter to dissect the pathogenesis of complex diseases such as MS but the international effort in understanding the disease and the aspiration to provide patients with better care is moving in the right direction. The future for

MS patients probably relies on ”precision medicine” with precise phenotyping of the patients first (in terms of their unique load of risks and dysfunction, and in terms of prediction of disease course and severity/treatment response) and targeted therapy attuned to their disease sub-phenotype.

Personal reflections:

During my years in the Neuroimmunology Unit I had to adapt to working with rats as I was working with mice during my masters. First, rats are way bigger than mice, which can seem very impressive at first, and second there is much less knowledge and reagents available to study immunology in rats than for mice or human cells. The first impression was easily changed since rats are very smart and relaxed animals, and handling them is far easier than handling mice. The second challenge was in the end very stimulating and I enjoyed establishing protocols for the passive EAE and bone marrow chimera techniques. This challenge also forced me to address not only complex questions with less conventional approaches but also to determine if some basic knowledge from mice held true for rats. Because this is an integrative multidisciplinary unit I had the opportunity to deepen my knowledge not only in immunology but in human and rodent genetic, epigenetic, stem cell therapy as well as to discover other models of neuroinflammation such as traumatic brain injury. It was a great opportunity to grow as an independent researcher in this group.

4. Acknowledgement

The beginning:

From: Marie N'diaye
Sent: den 9 juni 2010 16:35
To: Tomas.olsson@ki.se
Subject: Ph.D Position

Hello Dr. Olsson

My name is Marie N'diaye and I recently obtained my Masters degree from McGill University (Montreal, Canada). I was part of the department of immunology and worked in the laboratory of Dr. Fournier on a spontaneous murine model of neuroinflammation and demyelination. Our laboratory was part of the Montreal Neuroinflammation Training Program. I wish to pursue my career in the field of neuroimmunology. As a consequence I am looking for a PhD position.

I have attached to this email a copy of my C.V. and of my transcript.

I am a French citizen and am now based in Paris. You can join me at this email address (ndiaye_marie@hotmail.com) .

I am looking forward to hearing from you,

Cordially,

Marie N'diaye

From: tomas.olsson@ki.se
To: ndiaye_marie@hotmail.com
CC: Maja.Jagodic@ki.se
Subject: RE: Ph.D Position
Date: Thu, 10 Jun 2010 16:47:36 +0200

Dear Marie,

Your background is ideal for PhD thesis study here within our Neuroimmunology group.

The way we do it is usually to have a "testperiod" of 6-8 months during which the presumptive student works within an ongoing project together with advanced PhD students and post docs. We can offer a pay of about 1800 Euro per month during the testperiod. After this time, both the student and we consider the conditions and options for a definite registration for PhD thesis studies, which here is approximately four years. So far more than 80 % of persons doing this period in my group have decided to go on for this registration and PhD studies.

So if you are still interested please give me feedback.

Sincerely yours

Tomas

Tomas, I will forever be grateful for giving me the opportunity to join the Neuroimmunology Unit. Finding a PhD position was very difficult for me because of complicated relations with my previous supervisor and I was very close to giving up pursuing a career in research when you suddenly answered positively to my email. It was such a shock I had to ask my sister to read your email several times before I believed it! I will also always remember your words at my first group retreat when you said, I am paraphrasing, “all I want is for the people of this unit to be happy, because if they are happy they will do good research”. You have been nothing but encouraging and supportive during my time here and I think you succeeded in having a group where people are enjoying their work and colleagues!

Maja, you are the perfect example of a successful career woman in research! I secretly wanted to have a woman as my supervisor because… well I think they are smarter and kinder, and I didn’t want my previous experience to taint this idea. I cannot express how amazing it has been working with you and learning from you. You gave me enough support to move forward on the project but enough freedom to really learn how to become an independent scientist. Under your wings I felt competent and confident in my own capacity to reflect and progress. And I enjoy our non-scientific discussions as well about work/life social interactions and life in general. I wish you to continue to succeed in everything you undertake!

Andre, “Professor Cacais”, it’s hard for me to say how much I value our everyday interaction. You were here through it all!The successes, the doubts and the failures. You always had a good or harsh word to put me back on track and I am forever grateful for it. We laughed and cried together, discussed science, project, plans and dreams. I have no ideas how many times I came over to your office or called or sms you and your answer was always “tell me”, always ready to listen and guide. Andre, você é o melhor!

Bob, you are the wise man of the floor, always there to guide lost students back into the light. Thank you for our impromptu conversations about science, training or travel. Despite your busy schedule your door is always open and you have been crucial for dealing with the everyday administrative ordeal in research. You were the perfect completion to the dream team of supervisors!

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