Toward Second-Generation Cell Immunotherapies for Multiple Sclerosis: Combining Antigen Specific Approaches with Immunomodulation

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Doctoral Thesis

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Abbreviations

2-CdAMP: mononucleotide 2-chlorodeoxyadenosine 5 monophosphate

2-CdATP: triphosphorylated to 2-chlorodeoxyadenosine 5 triphosphate

ADA: adenosine deaminase

AHR: aryl hydrocarbon receptor

ALPS: Autoimmune Lymphoproliferative Syndrome

APCs: antigen presenting cells

APS-1: Autoimmune polyendocrine syndrome type-1

BCR: B cell Receptor

BBB: blood brain barrier

Bregs: regulatory B cells

cDCs: conventional DCs

CIS: clinically isolated syndrome

CMV: Cytomegalovirus

CNS: central nervous system

CSF: cerebrospinal fluid

DAMPs: damage-associated molecular patterns

DCs: Dendritic cells

DCK: deoxycytidine kinase

DMF: Dimethyl Fumarate

DMTs: disease-modifying therapies

EAE: Experimental Autoimmune Encephalomyelitis

EBNA1: EBV nuclear antigen 1

EBV: Epstein-Barr Virus

FDA: US Food and Drug Administration

GA: Glatiramer Acetate

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GlialCAM: glial cell adhesion molecule

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

GSDMD: gasdermin D

HCAR2: hydroxycarboxylic acid receptor 2

HHV-6: human herpes virus type 6

HIV: Human immunodeficiency virus

HLA: Human Leukocyte Antigen

HSCs: hematopoietic stem cells

HSV: Herpes Simplex Virus

IBD: inflammatory bowel disease

IDO: indoleamine 2,3-dioxygenase

IFNβ: Interferon β

ILCs: innate lymphoid cells

infDCs: inflammatory DCs

JCV: John Cunningham virus

KEAP-1: Kelch-like erythroid cell–derived associated protein-1

LPS: Lipopolysaccharide

mAb: monoclonal antibody

MBP: myelin basic protein

MCP-1: monocyte chemotactic protein-1

MHC: Major Histocompatibility complex

MMF: Monomethyl fumarate

mo-tolDCs: monocyte-derived tolerogenic dendritic cells

mDCs: mature dendritic cells

mTOR: mechanistic target of rapamycin

MS: Multiple Sclerosis

NAbs: neutralising antibodies

NCoR1: Nuclear receptor co-repressor 1

NF-κB: nuclear factor kappa-light-chain-enhancer

NK: Natural Killer

NMOSD: neuromyelitis optica spectrum disorders

Nrf2: nuclear factor erythroid 2–related factor 2

PAMPs: pathogen-associated molecular patterns

PBMCs: peripheral blood mononuclear cells

pDCs: plasmacytoid DCs

PD-L1: programmed death-ligand 1

PML: multifocal leukoencephalopathy

PPMS: Primary Progressive multiple sclerosis

PRRs: Pattern Recognition Receptors

pTregs: Peripherally induced Tregs

RA: rheumatoid arthritis

RRMS: relapsing-remitting multiple sclerosis

S1PR: Sphingosine-1-phosphate receptor

SLE: Systemic Lupus Erythematosus

SNP: Single-nucleotide polymorphism

SPSM: Secondary Progressive multiple sclerosis

T1D: type 1 Diabetes

TCR: T cell receptor

TEMRA: terminally differentiated effector memory

TGF-β: Transforming Growth Factor β

Th: T helper

tTregs: thymic Tregs

TNF-α: Tumour necrosis factor α

tolDCs: tolerogenic dendritic cells

TR1: IL-10-producing T regulatory type 1 cells

Tregs: T Regulatory Cell

VCAM-1: vascular cell adhesion molecule-1

1.Introduction

1.1 Immune Tolerance

The immune system is continuously patrolling the body to defend it against invading pathogens and maintaining a delicate balance to distinguish the “self” from the “non-self”. Specifically, the capacity to identify myriads of different antigens expressed univocally by the various types of microorganisms and parasites that can attack the organism represents the hallmark function of an evolved immune system. However, while this complex machinery can generate millions of different T and B cells able to recognize as many epitopes efficiently, it must also be able to ensure that no harm is done to self-structures.

Immune Tolerance is defined as the sum of a diverse range of processes actively preventing harmful immune responses against the “self”. Tolerance refers to a dominant, active, and highly regulated state where the immune system remains unresponsive to self-antigens or specific antigens that could potentially trigger an immune response in the body. In other words, tolerance can be described as a state of active unresponsiveness to an immunogenic antigen, which is unable to provoke an immune response. Immune tolerance enables the immune system to recognize and accept the body's tissues while still defending against external threats, maintaining a crucial balance to prevent autoimmune diseases, where the immune system erroneously targets its own cells and tissues. Various mechanisms support immune tolerance, ensuring the immune system does not react against the body's own components.

At the beginning of the 20th century, Paul Erlich described the event of the immune system to attack its own cells and tissues as “horror autotoxicus” (1).

Many years after, in 1989, Charles Janeway (2) proposed the “Infectious-Nonself model”, in which pattern-recognition receptors (PRRs) on antigen-presenting cells (APCs) recognise and destroy pathogens that show conserved pathogen-associated molecular-patterns (PAMPs) (infectious non-self), whereas do not recognize and attack self-components (non-infectious self).

One significant alternate conceptual theory for the understanding of immune tolerance is represented by Polly Matzinger's Danger Model (3) . The Danger Model, proposed by Matzinger in the mid-1990s, shifted this paradigm by suggesting that the immune system is more concerned with identifying danger signals rather than merely distinguishing between self and non-self (4).

According to the Danger Model, the immune system responds to "danger signals" emitted by cells undergoing stress, damage, or death, regardless of whether the cells are part of the body or are foreign. These danger signals can arise from a variety of sources, including infection, mechanical damage, or stress due to chronic inflammation. The model posits that immune responses are activated by the context in which antigens are encountered: antigens presented in the presence of danger signals trigger an immune response, while those encountered without such signals (1) in a context of cellular health and tissue homeostasis) are more likely to induce tolerance.

This model provides a more dynamic understanding of immune regulation, emphasising the context of antigen presentation as a critical factor in determining immune outcomes. The immune system, therefore, is seen as a highly adaptive and context-sensitive network that not only recognizes pathogens but also integrates signals from the tissue environment to decide whether to mount an attack or maintain tolerance.

In this sense, the Danger Model has profound implications for understanding autoimmune diseases (5,6), as it highlights the importance of controlling not just

the presence of antigens but the surrounding inflammatory context and signals that influence immune responses.

1.1.1 Central Tolerance

Central tolerance involves mechanisms that function during the development of immune cells in primary lymphoid organs, like the thymus for T cells and the bone marrow for B cells. This process eliminates self-reactive lymphocytes, technically ensuring only cells capable of recognizing foreign antigens with high specificity survive.

For T cells, central tolerance occurs in the thymus, a primary lymphoid organ located in the anterior upper mediastinum. Progenitors migrate from the bone marrow to the thymus, where T cell maturation and selection generate non-self-reactive naive T cells. Central tolerance begins with positive selection in the thymus cortex, where thymocytes that recognize low-affinity self-antigens presented by thymic epithelial cells receive survival signals, while those that do not undergo apoptosis in a process called "death by neglect." Positive selection ensures that T cells recognize Major Histocompatibility Complex (MHC) molecules. Following this, negative selection occurs in the thymus medulla, where dendritic cells (DCs) and medullary thymic epithelial cells present a variety of self-antigens. This is achieved through the activity of the transcription factor AIRE (Autoimmune Regulator), which promotes the expression of several genes encoding self-antigens (7).

T cells that strongly bind to self-antigens are eliminated through apoptosis, preventing harmful autoreactive T cells from entering the bloodstream.

However, some autoreactive T cells differentiate into naturally induced regulatory T cells (nTregs), which have suppressive functions and contribute to peripheral tolerance.

Central B cell tolerance takes place in the bone marrow, where immature B cells express a surface antigen receptor of the IgM class but have not completed maturation. Like T cells in the thymus, B cells undergo both positive and negative selection. Positive selection involves antigen-independent signalling via the pre-B-cell receptor (pre-BCR) and the B-cell receptor (BCR), requiring antigen receptor signalling for survival (8). If these receptors do not bind to their ligands, B cells start apoptosis. Developing B cells are positively selected when the pre-B receptor binds its ligand, ensuring the BCR is functional and capable of recognizing antigens. Negative selection induces apoptosis when the BCR strongly binds to self-antigens in the bone marrow, preventing the survival of self-reactive B cells (9).

Despite the existence of central tolerance, not all self-reactive T and B cell clones generated in the thymus and bone marrow are successfully eliminated. Indeed, a small fraction of self-reactive immune cells may escape to the periphery because of stochastic errors in central tolerance, posing a risk for autoimmune responses. Moreover, not all possible autoantigens are presented in the thymus, meaning that additional control systems need to be enforced to avoid autoreactivity against self-tissues. Finally, the escape of low-affinity self-reactivity can be justified to provide a wider T cell repertoire.

1.1.2 Peripheral Tolerance

During evolution, the immune systems developed an additional system operating outside the primary lymphoid organs allowing it to maintain immune tolerance in the periphery. This blocks the autoreactive responses operated by clones that escape deletion in the primary lymphoid organs, as well as by clones with low affinity toward self-antigens that are not negatively selected. Peripheral tolerance comprises a set of mechanisms that act outside the primary lymphoid organs and function to control potentially harmful autoimmune responses in the periphery. Among the hallmark mechanisms of peripheral tolerance, the most well-studied include the induction of an unresponsiveness stat called “anergy” in autoreactive T cells, the generation of Tregs that suppress immune responses to self-antigens in the periphery a peripheral deletion through activation-induced cell death mediated by immature DC (10).

Specifically, anergic T cells are not able to activate, expand and release cytokines upon encountering their cognate antigens. This state can be induced by co-inhibition or excessive signalling through the TCR without sufficient co-stimulatory signals. These conditions arise during chronic exposure to antigens in the absence of appropriate co-stimulatory molecules, a scenario commonly observed in persistent infections or cancer. In this sense, induction of anergy is necessary to dampen exaggerated immune responses and avoid toxicity and autoimmunity during extended inflammatory responses.

On the other side, Tregs are a unique type of T-cells involved in inducing tolerance, characterized by the expression of FoxP3, CD25, CTLA4, and negative/low CD127 (11).

nTregs develop in the thymus from CD4 single positive thymocytes that can recognize autoantigens and escape positive selection. These nTregs exit the

thymus and induce tolerance in the periphery through various mechanisms. Besides nTregs, another subset of Tregs called peripherally induced Tregs (pTregs) is generated outside the thymus. pTregs arise from conventional CD4+ T cells through peripheral induction in response to specific environmental factors, such as antigen exposure and cytokine signalling (11).

Both nTreg and pTregs are essential and not redundant for maintaining peripheral tolerance, achieved by suppressing the activation and functions of other immune cells. Tregs modulate both innate and adaptive immunity through various mechanisms, as per the secretion of immunomodulatory cytokines such as Interleukin-10 (IL-10), Transforming Growth Factor beta (TGF-β), and Interleukin-35 (IL-35) (12).

IL-10, a critical regulatory cytokine, dampens both innate and adaptive immune responses by activating STAT3 and controlling the expression of anti-inflammatory genes (13,14). IL-10 is a pleiotropic cytokine and among its many effects, it can downregulate the production of Th1 cytokines, MHC class II antigen presentation, decrease expression of co-stimulatory molecules on APCs, inhibit NF-kB signalling and induce antibody production in B cells and inhibition of NF-kB signalling.

TGF-β plays a pivotal role in regulating both adaptive and innate immunity and is vital for stem cell regulation and differentiation. It directly inhibits T-cell activation, differentiation, and proliferation (15). Notably, TGF-β can also contribute together with retinoic acid to transform naïve T cells into T regulatory cells known as induced Tregs (iTregs) (16,17).

IL-35, another regulatory cytokine produced by Tregs, is crucial for their full suppressive function and, like TGF-β, can also induce the formation of other Tregs (18).

In addition to producing immunosuppressive cytokines, Tregs induce tolerance through Perforin/Granzyme-dependent cytolysis (19) and by causing metabolic

disruption of other immune cells via IL-2 deprivation (20). Finally, Tregs can directly interact with DCs, reducing their ability to activate effector T-cells by inducing the expression of immunosuppressive molecules such as indoleamine 2,3-dioxygenase (IDO), programmed death-ligand 1 (PD-L1), CTLA-4, among the many (21,22).

1.2. The Emergence of Autoimmunity

In homeostatic conditions, the immune system can maintain a balance between immunity against the non-self and tolerance against self-antigens. However, when this balance is altered, autoimmunity can occur.

Autoimmunity is a complex system of processes leading to the breakdown of self-tolerance, arising from the interplay of genetic, environmental, and immunological factors. While the precise triggers and mechanisms can vary between different autoimmune diseases, several common elements underlie the emergence of autoimmunity.

Firstly, most autoimmune diseases have a genetic component, so individuals with a family history of such conditions are more likely to develop them. Several genes related to immune regulation and self-tolerance can increase the risk of autoimmunity. For example, in diseases like rheumatoid arthritis (RA), Systemic Lupus Erythematosus (SLE), and type 1 Diabetes (T1D), over 100 different genetic loci are linked to an increased risk of these diseases. Autoimmune diseases are considered multigenic, and susceptibility arises from a combination of specific susceptibility nucleotide polymorphisms (SNPs) (23). Generally, polymorphisms in MHC genes are the strongest risk factors for several autoimmune diseases, including RA, SLE, MS, and T1D (23).

However, for most autoimmune diseases, genetic predisposition alone is insufficient to cause autoimmunity, highlighting the importance of additional factors. Nevertheless, specific mutations in key genes can directly lead to autoimmunity, such as gain-of-function mutations in TLR7 causing monogenic SLE (24), mutations in the AIRE gene causing Autoimmune Polyendocrine Syndrome type-1 (APS-1) (25) (19), in FoxP3 in Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX syndrome) (26) or mutations in the Fas gene causing Autoimmune Lymphoproliferative Syndrome (ALPS) (27). Additionally, autoimmune diseases are more prevalent in women than in men, indicating sex as a significant genetic risk factor or female sex hormones as linked to autoimmunity (28–32). Thus, while genetic predisposition significantly influences the likelihood of developing autoimmune diseases, it is not sufficient to cause them in most cases, as studies on identical twins suggest. Indeed, identical twin studies show only about 50% concordance in T1D (33) with similar results observed in SLE (34), indicating that additional factors contribute to the onset of autoimmune diseases.

Apart from genetics, environmental factors, such as infections, diet, and exposure to toxins can play a pivotal role in triggering and sustaining autoimmunity. Among environmental factors, infections appear to be the most common trigger of autoimmunity (35). Specifically, it has been hypothesized that microorganisms trigger autoimmunity through molecular mimicry based on structural similarity between self and foreign antigens.

In this context, lymphocytes start recognizing self-antigens that are structurally like the ones expressed by the microorganism, starting to target self-tissues through a process of cross-reactivity.

This mechanism has been proposed in the context of MS (36), in which infection with the Epstein-Barr Virus (EBV) was described to be associated with B-cells producing cross-reactive antibodies against the EBV transcription factor EBV

nuclear antigen 1 (EBNA1) and the host central nervous system protein glial cell adhesion molecule (GlialCAM). Another example of autoimmune manifestation is rheumatic fever, in which infection with *Streptococcus pyogenes* can trigger production of antibodies that cross react with heart and joint tissues (37). Autoimmune manifestations that do not culminate in autoimmune diseases can also be induced by nonspecific stimulation of the immune system, and in particular, of the innate one. Indeed, PAMPs molecules such as endotoxin (LPS) and superantigens can overly stimulate the innate immune system and eventually lead to excessive activation of T and B cells, potentially resulting in toxicity and the onset of autoimmune symptoms.

Moreover, host microbiota composition is strongly linked to the risk of developing certain autoimmune diseases (35). For instance, high levels of Immunoglobulin A coating of gut bacteria are associated with Inflammatory Bowel Disease (IBD) development (38). Similarly, Aggregatibacter actinomycetemcomitans, a periodontal commensal fungus, can induce citrullination of autoantigens and neutrophil activation in RA patients via the toxin leukotoxin A (39). Conversely, the host microbiota is crucial in regulating the immune system and promoting tolerance to bacterial and host antigens, thus protecting against autoimmune responses (40,41). Dietary regimens also play a role in autoimmune disease development due to their significant impact on microbiota, mucosal permeability for commensals and inflammatory status (42,43). Additionally, xenobiotics have been suggested to facilitate the onset of various autoimmune diseases (44).

Taken together, autoimmune diseases appear to have a complex and multifaceted aetiology, which is rarely explained by only genetic or environmental factors.

1.2.1 Autoimmunity as a breakdown of immune tolerance

Despite the existence of varying causes contributing to triggering an autoimmune disease, a central theme in autoimmunity is indeed the loss of immune tolerance. In fact, autoimmunity can be defined as a state of loss of tolerance of self-antigens in the presence of a pathogenic process, with different clinical manifestations according to the type of self-responses involved. This breakdown in tolerance is often due to multiple failures in the regulatory mechanisms that keep the balance of the immune system, which are determined by genetic elements and environmental factors that eventually end up affecting the physiological processes that characterise the immune system.

This failure in tolerance can occur at multiple levels. First, there can be a failure at the level of central tolerance. Indeed, even in healthy organisms, not all autoreactive T and B cell clones are eliminated during development. This eventually leads to medium to high-affinity autoreactive clones that reach the periphery, where they can be activated by their cognate self-antigen. However, in addition to central tolerance, peripheral tolerance mechanisms evolved to ensure that self-reactive clones that escape positive selection are eliminated or cannot unleash their effector function and cause damage to the organism. As mentioned earlier, these mechanisms encompass Tregs, which promote tolerance by various means to suppress autoreactive lymphocytes. Additionally, immune checkpoints are molecules expressed by lymphocytes that serve as guardians of the immune system, modulating immune responses to prevent excessive attack on healthy cells (45). However, inflammation can disrupt immune tolerance by creating an environment where immune cells become more reactive and less discriminating. This can be triggered by infections, tissue damage, or other inflammatory stimuli. Additionally, environmental factors like

smoking, diet, and stress can contribute to a pro-inflammatory state that promotes autoimmunity (46,47).

When these processes fail, autoreactive immune cells can become fully active and trigger autoimmunity. In summary, autoimmunity develops through a complex interplay of genetic susceptibility, environmental triggers, and the breakdown of immune tolerance mechanisms.

1.3 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic autoimmune and inflammatory condition affecting the central nervous system (CNS). It is characterised by an autoimmune response targeting the myelin sheath that surrounds axons of neurons.

It is the leading cause of non-traumatic disability among young adults (48) and affects over 2.5 million people globally (49). The risk for MS varies by sex, with women being twice as likely to develop MS as men, and it most commonly manifests in young adults aged 20 to 30 years, with a global prevalence of 35.9 per 100,000 people (50). However, prevalence differs significantly by region, from 108 cases per 100,000 people in Europe to fewer than 4 cases per 100,000 people in the Western Pacific (51,52). Interestingly, MS is more frequent north of the equator, possibly due to different HLA distribution and lower sunlight exposure, as vitamin D is considered a protective factor (53).

1.3.1 Clinical Aspects of MS

MS is a disease characterised by highly heterogeneous clinical manifestations among different individuals. Symptoms associated with MS can be

unpredictable and are closely tied to the specific areas of the CNS affected by demyelination. This variability makes the clinical aspects and symptomatology somewhat unreliable for diagnostic purposes (54). However, cognitive impairment is a prevalent characteristic across all types of MS and can emerge early in the natural history of the disease (55). The diagnostic criteria for MS have been originally established by McDonald in 2001, and have undergone updates and changes in 2005, 2010, and 2017 (56). Accurate diagnosis needs objective CNS injury and often requires additional information regarding the dissemination of the disease "in space and time" (57).

Number of attacks	Number of lesions	Additional requirements for MS diagnosis
≥2	≥2	None
≥2	1	Clear evidence of previous attack with a lesion in a distinct anatomical location
≥2	1	Dissemination in space: 1) An additional clinical attack at a different CNS site OR 2) MRI evidence of T2-weighted lesions in ≥2 of 4 areas (periventricular, cortical/juxtacortical, infratentorial, and spinal cord)
1	≥2	Dissemination in time: 1) An additional clinical attack OR 2) MRI evidence of gadolinium enhancing and nongadolinium enhancing lesions OR 3) CSF oligoclonal bands
1	1	Dissemination in space AND dissemination in time
0	1	>1 year of disease progression AND 2 of the following: 1) T2-weighted hyperintense lesion in 1 of 3 brain areas (periventricular, cortical/juxtacortical, and infratentorial) 2) T2-weighted hyperintense lesion in ≥2 spinal cord areas 3) Oligoclonal bands

Figure 1: Revised 2017 McDonald criteria for the diagnosis of MS (58)

In general, it is possible to differentiate between four primary types of MS (59):

1. **Clinically Isolated Syndrome** (**CIS**): CIS refers to the initial manifestation of neurologic symptoms caused by inflammatory responses and demyelination which align with an MS diagnosis but do not yet meet the criteria for dissemination in time. While not all individuals with CIS develop MS, those with CIS have a high likelihood of experiencing a second episode of neurologic symptoms and being diagnosed with MS. Symptoms of CIS vary but often include vision

problems, vertigo, loss of sensation, limb weakness, coordination and balance difficulties, walking, speaking, and swallowing issues, as well as bladder problems.

2. **Relapsing-Remitting MS** (**RRMS**): RRMS represents the most common type of MS, accounting for 85% of MS cases. It is characterised by temporary periods of relapses or exacerbations when symptoms appear (relapsing or active disease phase), followed by periods of remission when the symptoms disappear or subside (non-active/remission phase). Specifically, in the event of a relapse, the individual undergoes a period of heightened symptoms related to inflammatory demyelination, lasting for a minimum of 24 hours. After a new relapse, new symptoms may appear or existing ones may worsen, while remission periods entail a partial or complete recovery from the symptoms. RRMS mostly affects individuals between 20 and 40 years old, with women being more likely to be diagnosed with this form of the condition.

3. **Primary Progressive MS** (**PPMS**): PPMS is a type of MS characterized by a continuous functional decline from the beginning of the illness. Unlike relapsing-remitting MS, which involves periods of relapse and remission, PPMS is marked by a continuous worsening of symptoms. Approximately 10% of people with MS are diagnosed with this type of disease. Despite the similarities between PPMS and relapsing-onset MS, there are also differences, including age of onset and the response to treatments. For example, PPMS usually affects older individuals and is equally prevalent in men and women (60).

4. **Secondary Progressive MS** (**SPMS**): SPMS is marked by a transition from an initial relapsing-remitting pattern to a more steadily progressive

phase, with or without sporadic relapses, small remissions and periods of plateaus. In other words, this type of MS follows an initial period of relapsing-remitting MS that eventually evolves in a progressive form. In SPMS, the disease may still have relapses or not, but the disability progressively worsens over time. Eventually, up to two thirds of patients with RRMS develop SPMS (61).

1.3.2 Aetiology

Like other autoimmune diseases, MS is not considered hereditary, although specific genetic factors strongly contribute to the risk of developing it. Previous studies suggest that genetic variability accounts for up to 30% of the risk of developing MS, while external factors are the most important risk factor, accounting for up to 70%. MS appears to be a polygenic disease, with more than 200 genetic MS-associated SNPs having been identified (62). Some of them, like those located in Human Leukocyte Antigen (HLA) class II alleles DRB1*1501, DRB1*0301, and DRB1*1303, and the genes of the α-chains of the IL-7 and IL-2 receptors (IL-7Rα and IL-2Rα) are linked to a higher risk for MS (63,64), while other genes seem to be linked with a decreased risk, like HLA class I allele A2 (65).

Different pathogens, like EBV, human herpes virus type 6 (HHV-6) and Mycoplasma pneumoniae, have also been associated with the onset of MS (66). It has been hypothesised that these pathogens may present molecular mimicry to myelin (67), causing T cells to be prone to unwanted activation by myelin antigens, thus becoming autoreactive. Specifically, a recent study analysing the prevalence of MS in a cohort of American soldiers evidenced a strong correlation between EBV infection and MS, suggesting that infection from this virus to be one of the leading causes of MS (68).

As previously mentioned, this association has been explained mechanistically through a mechanism of molecular mimicry determined by the structural similarity among EBNA1 viral protein and the glial protein GlialCAM (36). Meningeal tertiary lymphoid-like structures composed of EBV-infected B-cells have also been described in progressive MS patients (69).

Moreover, infections can induce high levels of peripheral inflammation which might have a strong impact on the CNS and MS onset and exacerbation. Indeed, proinflammatory cytokines released after exaggerated activation of the innate immune system can travel across the blood-brain barrier (BBB), leading to microglia and macrophage activation and CNS inflammation (70,71)**.** Other predisposing factors include smoking, deficiency of vitamins (especially vitamins D and B12), diet, exposure to ultraviolet light and childhood obesity (49).

1.3.3 Pathogenesis and immune landscape of MS patients

There is a consensus that acknowledges that the disease's pathogenic mechanism of MS starts with an escalated migration of activated self-reactive CD4 T-cells traversing the BBB into the CNS, where they initiate an immune response within the brain. Within this context, regulatory lymphocytes, whose function is compromised (72), are unable to suppress autoreactive T cells. Consequently, local APCs reactivate autoreactive T cells, causing the detriment of myelin, oligodendrocytes, and nerve fibres, eventually resulting in damage and inflammation.

However, there is uncertainty surrounding the mechanism behind the onset of the disease, which has led to the stipulation of two distinct models of MS pathogenesis. In the ‘CNS-intrinsic model’, the initiation of disease occurs

through intrinsic CNS events, with lymphocytic infiltration only taking place in response to this primary trigger. However, the specific intrinsic events within the CNS that lead to disease development remain unidentified, with viral infections being one of the candidate causes (73) together with processes leading to primary neurodegeneration. Conversely, in the 'peripheral onset model', autoreactive CD4+ T cells become activated by APCs. This activation can occur for various reasons, like pathogenic molecular mimicry (as mentioned earlier), bystander activation (activation of CD4+ T cells without antigen recognition), as co-expression on activated cells of T cell receptors with different specificities, enabling simultaneous recognition of self and viral antigens (73,74). Independently of the model, in MS a part of the activated CD4+ T cells will differentiate into T helper (Th) 1 cells or Th17 cells, depending on the cytokine signalling that they receive from APC. These cells then enter the bloodstream, and after upregulating specific integrins like VLA-4 (75) cross the BBB and eventually reach the central nervous system.

B cells and monocytes also cross the BBB at this stage, contributing to hindering BBB selectivity and fueling CNS inflammation.

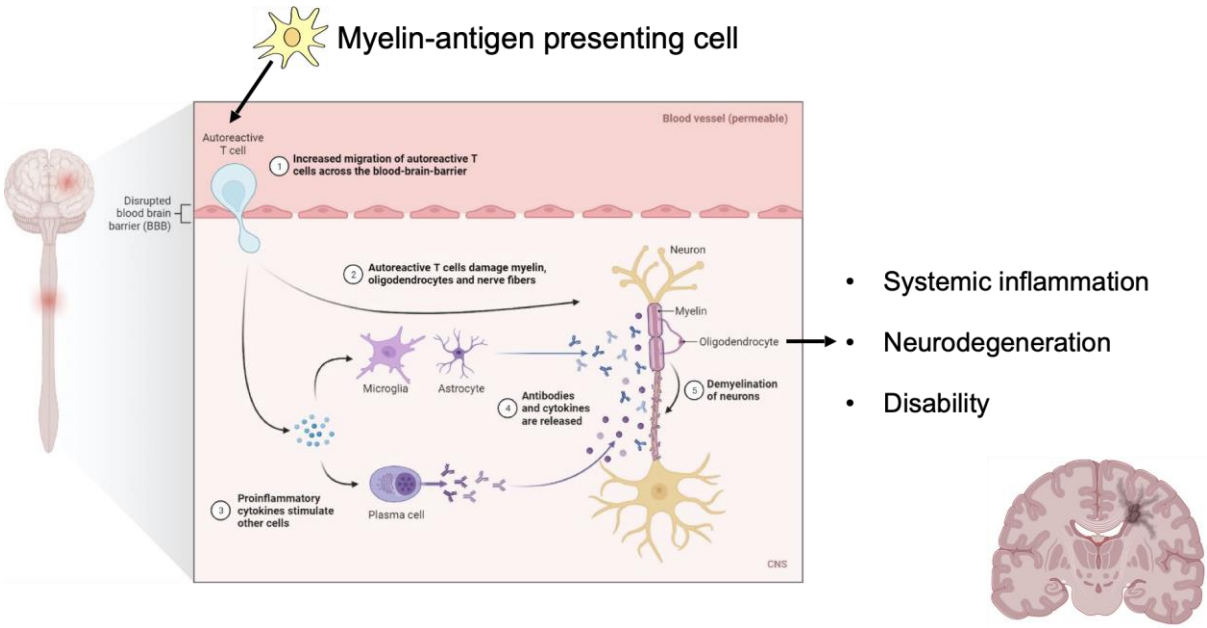


Figure 2. MS pathogenesis model (created with Biorender)

The release of proinflammatory cytokines both in the periphery and within the CNS initiates the activation of microglia and astrocytes, inducing the migration of more inflammatory cells, and stimulating antibody production by plasma B cells (76,77). This inflammatory environment further drives the progression of MS by generating more proinflammatory cytokines, as well as oxygen and nitrogen free radicals, establishing a cyclical pattern of inflammation and oxidative stress (78,79). Inflammation primarily contributes to damage of oligodendrocytes and demyelination, with axons initially being relatively preserved during the early stages of RRMS when inflammation is predominant (80,81). However, as the disease progresses to SPMS, irreversible axonal damage occurs, ultimately leading to disability.

During the acute/relapsing phases of MS, when there is BBB leakage, CD8+ and CD4+ T cells, B cells, and macrophages play crucial roles in attacking myelin, leading to demyelination. CD8+ cells, characterised by a tissue-resident memory phenotype, undergo in situ CNS clonal proliferation and exhibit signs of activation, indicating local recognition of antigens (82). The precise pathogenic role of B cells remains partially understood, but B cells compartmentalised within the CNS may contribute to MS progression by producing autoreactive antibodies that target oligodendrocytes and astrocytes, as well as through non-antibody-dependent mechanisms such as antigen presentation, T cell activation, and cytokine production (83).

Inflammatory infiltrates can lead to focal areas where the protective myelin sheath is lost, often accompanied by varying degrees of damage to nerve fibres. This process is primarily driven by activated microglia and macrophages. After the initial autoimmune attack, immune cells within the CNS tissue undergo programmed cell death, while macrophages and microglia may adopt an anti-inflammatory or reparative role (84).

During the progressive stages of MS, a distinct pattern of inflammation emerges. There is reduced leakage across the blood-brain barrier (BBB),

allowing T and B cells to gradually accumulate in the brain and spinal cord tissue, particularly affecting the meninges and areas around the brain's ventricles. Notably, these infiltrating cells organise into localised clusters resembling tertiary lymphoid follicles. CD8+ T cells often exhibit a resident memory phenotype with activation localised to specific regions (84). In chronic lesions, the predominant B cell types are plasmablasts and plasma cells. Tissue damage involves activation of microglia and macrophages, oxidative stress, and dysfunction of mitochondria. This ongoing inflammation correlates with the enlargement of existing lesions and widespread degeneration in visibly unaffected areas of white or grey matter (85,86).

Several studies indicate that this secondary type of inflammation appears early in the progression of MS and becomes more pronounced as the disease advances with age. The inflammatory response, initiated by the infiltration of immune cells from the peripheral blood and innate immune cells residing in the CNS, may hasten ageing processes, leading to significant and progressive neurodegenerative decline. This cascade of inflammation also contributes to the formation of demyelinating plaques, which are primarily concentrated in the white matter surrounding the brain's ventricles, cerebellum, optic nerves, brainstem, and throughout the spinal cord. Damage to these CNS structures results in typical MS symptoms such as muscle weakness or stiffness, numbness in limbs, balance issues, visual disturbances, and cognitive impairment (54). While many lesions eventually show signs of remyelination, the original thickness of the myelin sheath never fully recovers, resulting in persistent disability (49).

1.3.4 Insights from the EAE mouse model

A significant portion of our knowledge about the development of MS has been gained through investigations using different mouse models. The Experimental Autoimmune Encephalomyelitis (EAE) model is the most used animal model and deserves a detailed explanation. This model mimics various clinical and histopathological aspects of MS and is commonly induced by either actively immunising animals with myelin peptides together with an adjuvant or by transfer of primed myelin-specific CD4+ T cells. In this model, the pathological process begins with an influx of immune cells from the periphery into the meninges several days before the detection of inflammatory cells in the CNS. Myelin antigens eventually move from the CNS to the meninges through the cerebrospinal fluid (CSF), where they are presented by perivascular macrophages or microglial cells to T cells. Antigen presentation within the CNS triggers the reactivation of T cells specific to myelin, initiating an intricate immune reaction that engages diverse cell types, such as B cells, NK cells, macrophages, and innate immune cells (87). This sequence results in the generation of proinflammatory cytokines, along with reactive oxygen and nitrogen species (ROS and RNS), which contribute to the breakdown of the BBB, enabling cell infiltration into the CNS parenchyma. This cascade leads to inflammation around blood vessels, demyelination, and neuronal harm (87).

However, MS is a disease affecting only humans, and several disparities emerge when comparing the immunological characteristics of MS with the ones displayed by the different EAE mouse models, especially evident when analysing the immune cell composition found in MS lesions. While CD4+ T cells are typically associated with EAE, CD8+ T cells are more prevalent in MS lesions. CD8+ T cells in MS lesions are implicated in direct attacks on oligodendrocytes, leading to apoptosis, and in the damage to neurons through the release of cytotoxic granules, ultimately contributing to axonal degeneration.

Notably, a subset of CD8+ T cells in active lesions has been identified as capable of producing IL-17 and its depletion appears to be related to good response to the treatment with the drug dimethyl fumarate (88), suggesting their potential role in disease pathogenesis.

Apart from T-cells, recent studies have revealed an increased recognition of the pathogenic function played by B cells in MS (89,90). Clonally expanded B cells can be located in various compartments, including the CSF, parenchyma and meninges. Moreover, these B cells produce immunoglobulins within the CNS in an oligoclonal pattern. Indeed, ectopic lymphoid follicles can be present in proximity to demyelinating lesions in the meninges of MS patients and they have been linked to disease severity, highlighting that B cell maturation can be perpetrated locally (87). Finally, the role of B cells in MS is supported by the real-world clinical evidence represented by the therapeutic success that anti-CD20 drugs have in the treatment of MS patients (91).

There is increasing knowledge that innate cells from both myeloid and lymphoid lineage also play an important role in MS pathogeny. Indeed, DCs, macrophages, natural killer (NK) cells, and innate lymphoid cells (ILCs) resident in the meninges are increasingly recognized for their involvement in MS pathogenesis. These cells, through various mechanisms, influence both the initiation and progression of the disease. Mast cells, for instance, contribute to BBB permeability and inflammation through the production of cytokines, while ILCs exhibit phenotypic plasticity analogous to T cells, playing diverse roles in the inflammatory responses associated with EAE (87).

The intricate involvement of all these different immune cell subsets emphasises the complexity of MS pathogenesis, extending beyond the traditionally implicated T and B lymphocytes. Furthermore, it is important to point out that certain cell populations involved in tolerance induction and immune suppression, like Tregs, regulatory B cells (Bregs), tolerogenic DCs (tolDCs), and regulatory CD56hi NK cells, can be altered in MS. In fact, dysregulation of these regulatory mechanisms may fuel, and not dampen, the pathogenic functions of the immune system of an MS patient. Indeed, MS-specific aberrant immune response is constrained by the activation of complex anti-inflammatory processes, including apoptosis of inflammatory cells, release of anti-inflammatory cytokines, and clearance of debris and dead cells. However, several studies point out numerical reduction and functional defects in FoxP3 expressing CD4+ Tregs (72,92) and T regulatory type 1 Reg (Tr1), characterised by the production of IL-10 (93).

1.3.5 Treatments approved for MS patients: immunosuppressants and immunomodulators

MS is a disease with high prevalence and a devastating effect on the quality of life of patients affected by this disease. For these reasons, there have been, and still are, enormous efforts to find therapeutic options. A significant breakthrough took place in 1993 when interferon beta (IFNβ) was approved as the first therapy for RRMS patients (94). Since then, the treatment paradigm for individuals diagnosed with MS has undergone a dynamic transformation, reflecting the incessant pursuit of more efficacious and precisely targeted therapeutic interventions. Specifically, the field of MS therapeutics has witnessed significant advancements, with a plethora of treatment options

emerging to address various aspects of the disease spectrum and allowing clinicians to choose among more than 10 first and second-line treatments (57).

Central to the current therapeutic landscape are conventional disease-modifying therapies (DMTs), which try to modify the course of MS by suppressing the aberrant immune responses responsible for neuroinflammation and demyelination. These therapies, ranging from interferons to monoclonal antibodies, represent a cornerstone in the management of RRMS. However, the variable response rates and potential side effects associated with DMTs underscore the ongoing need for refining treatment approaches and developing personalised strategies.

Despite having been in the market since the 90s, IFNβ pharmacodynamics has not been completely elucidated. IFNβ is a naturally occurring cytokine produced by both cells belonging to the adaptive and innate immune system. Overall, it exerts a conspicuous range of anti-inflammatory, immunomodulatory, antiviral, and antiproliferative effects (95). From a clinical point of view, its impact includes lowering MRI lesion activity, reducing brain atrophy, extending the time to clinically confirm MS, and decreasing relapse rates (96). At the level of immune cell function, in MS patients IFNβ can directly increase the expression of anti-inflammatory agents, decrease the trafficking of inflammatory cells through the BBB, and enhance nerve cell survival and repair. Moreover, IFNβ can prevent T cell activation through the downregulation of MHC II expression in APCs (97). Additionally, it can increase the number of blood regulatory, CD56hi natural killer cells, which can produce anti-inflammatory cytokines (98). Overall, all these effects are thought to contribute to the overall therapeutic benefit of IFNβ in MS, making it a widely used first-line treatment for relapsing forms of the disease (96,99,100). The use of IFNβ in MS has been well established, and several formulations of IFNβ are available for the treatment of relapsing forms of MS including subcutaneous IFNβ-1 and others. IFNβ is immunogenic, and has been shown to cause allergic reactions (101).

Moreover, the use of IFNβ can induce formation of neutralising antibodies (NAbs), which can diminish the effectiveness of IFNβ preparations, and lead to a deterioration in disease outcomes (102). Common adverse events encompass influenza-like symptoms, headaches, leukopenia and lymphopenia, thyroid disorders, autoimmune reactions, depression, and elevated liver enzymes, with the potential for severe hepatic injury all of which are commonly documented (103).

Since the introduction of IFNβ, the treatment options for MS have broadened to comprehend a variety of novel drug targets and disease-modifying therapies.

The second drug to be approved for RRMS and CIS was an immunomodulator called Glatiramer Acetate (GA), in 1996. GA consists of random-sized peptides comprising the four amino acids present in myelin basic protein (MBP) (L-alanine, L-lysine, L-glutamic acid, and L-tyrosine) that are presented to myelin-specific CD4 T-cells by the patient's APCs MHC class II molecules (104). Even if the exact mechanism of action of GA is not clear, it has been described that GA may exert its therapeutic action by competing with myelin antigens for binding to MHC class II (105), thus acting as a decoy target to the autoreactive immune system and deviating the myelin attack. Furthermore, studies have demonstrated that the administration of GA leads to the development of a subset of CD4+ Th2 cells specifically responsive to GA in the peripheral immune system (106), as induction of functional FoxP3+ Tregs (107). Even though GA is generally well tolerated, up to 10% of patients treated with this drug experience immediate systemic reactions characterised by flushing, chest pain, and palpitations (108). Additional frequent side effects include reactions at the injection site, such as redness, pain, swelling, and lipoatrophy.

In 2000 Mitoxantrone was approved, resulting in the first drug specifically available for progressive MS forms. Mitoxantrone is a synthetic anthracenedione derivative administered to SPMS and severe RRMS patients

(109). It is an antineoplastic and cytotoxic drug that works by intercalating in DNA and causing strand breaks which eventually lead proliferating cells to die. Thus, mitoxantrone seems to work by mainly inducing cell lysis and initiating programmed cell death in proliferating B and T lymphocytes. However, several studies have also pointed out the immunomodulatory effects of this drug. Specifically, Mitoxantrone was shown to reduce B cell functionality (110) and the migratory ability of monocytes into CNS while promoting increased production of Th2 cytokines in CD4+ T cells (111). Moreover, In vitro studies indicate that Mitoxantrone hinders the antigen-presenting capabilities of DCs (103). As for side effects, mitoxantrone's use is associated with potential cardiotoxicity, including dose-related cardiomyopathy and congestive heart failure. Other typical adverse effects encompass nausea, hair loss, menstrual irregularities, and heightened susceptibility to infections. Mitoxantrone's use is restricted due to its potential for cardiotoxicity, necessitating limits on lifetime cumulative dosage and mandatory cardiac monitoring (109).

In 2004 the first monoclonal antibody (mAb) for the treatment of RRMS was approved. Natalizumab, a humanised IgG4κ mAb, works by selectively binding through allosteric antagonism to α4-integrin (CD49d) (112). Specifically, Natalizumab binds to the α4 subunit of α4β1 and α4β7 integrin receptors present on the outer layer of the plasma membrane of lymphocytes, thereby blocking the α4-mediated adhesion of leukocytes to their ligands. Indeed, on the surface of leukocytes, α4β1 (VLA-4) and α4β7 engage with VCAM-1 and MAdCAM-1, correspondingly, facilitating the adhesion of leukocytes to endothelial cells and allowing extravasation and migration to inflamed tissues, as crossing of the BBB. Thus, Natalizumab exerts its main mechanism of action by drastically reducing the migration of lymphocytes through the BBB into the CNS (113). Blocking extravasation results in the accumulation of mononuclear cells in circulation, which correlates with an elevated count of circulating lymphocytes (114). Moreover, Natalizumab treatment has been shown to

decrease plasma and CSF levels of osteopontin in RRMS patients (115). Several studies confirm that natalizumab is an efficacious treatment option for RRMS patients and that it is more effective than other DMTs like IFNβ and GA (116). However, despite its benefits, Natalizumab was withdrawn from the market in 2005 due to reports of two cases of progressive multifocal leukoencephalopathy (PML), a demyelinating disease of the CNS caused by the reactivation of the John Cunningham virus (JCV), leading to the lytic infection of oligodendrocytes. While up to 58% (117) of the general population is seropositive for JCV, only immunocompromised individuals develop the disease. In the case of MS patients receiving Natalizumab, PML is thought to be due to the reduced migration of lymphocytes in the CNS, which compromises physiological immune surveillance and facilitates reactivation of the virus (118). Natalizumab was then reintroduced in 2006 by FDA with new labelling and safety warnings clarifying the risk of PML, which has been calculated for 4 every 1000 patients (119,120).

In 2010 Fingolimod, the first Sphingosine-1-phosphate receptor (S1PR) modulator, was approved for RRMS. Fingolimod phosphate, the active form of the drug, closely resembles endogenous Sphingosine-1 Phosphate, an extracellular lipid mediator that primarily exerts its effects through specific G protein-coupled receptors. Among these receptors, termed S1P1-5, four are capable of binding fingolimod phosphate (121). S1P1 plays a critical role in the immune system by governing the exit of lymphocytes from lymphoid tissues into circulation. Initially, fingolimod phosphate activates S1P1 on lymphocytes through high-affinity binding to the receptor. However, it subsequently prompts internalisation, degradation, and down-regulation of S1P1 (122). This process involves the elimination of S1P1 from cell membranes, rendering lymphocytes unresponsive to the S1P gradient. This prevents their normal exit from lymphoid tissues and circulation to the periphery. Consequently, most lymphocytes, including autoreactive T and B cells, are sequestered in lymph nodes, unable

to migrate to the CNS to attack myelin and perpetuate inflammation (123). Following Fingolimod, another S1PR modulator called Siponimod was approved in 2015. Fingolimod acts as an agonist on four out of the five S1P receptors (S1P1, S1P3, S1P4, and S1P5), while Siponimod selectively targets S1P1 and S1P5, making it potentially more tolerable in terms of side effects and likely less immunosuppressive (124). Additionally, Ozanimod and Ponesimod were approved in 2020 and 2021, respectively. Ozanimod targets S1P1 and S1P5, whereas Ponesimod is specific to S1P1.

In 2012 Teriflunomide was approved for the treatment of RRMS, and later of CIS and active SPMS. Teriflunomide seems to mainly exert its mechanism of action by inhibiting pyrimidine synthesis through blockage of the enzyme dihydroorotate dehydrogenase, thus reducing T and B cell activation and proliferation (125). Moreover, Teriflunomide seems able to suppress interleukin 1 (IL-1) and tumour necrosis factor α (TNF-α) selectively in T lymphocyte/monocyte contact activation (126), as to inhibit the formation of the immune synapse between APCs and T-cells. Finally, in vitro studies showed that Teriflunomide reduced the production of IL-8, IL-6 and monocyte chemotactic protein-1 (MCP-1) in activated peripheral blood mononuclear cells (PBMCs) (125). The most common adverse events associated with teriflunomide include increases in liver enzyme levels, diarrhoea, and potential teratogenicity. For this, teriflunomide can’t be used during pregnancy. Other reported side effects are peripheral neuropathy, hypertension, nausea, and lymphopenia.

In 2013, just one year after Teriflunomide, the drug Dimethyl Fumarate (DMF) was approved for the treatment of RRMS. DMF, also known as Tecfidera, is a second-generation fumaric acid ester approved for the treatment of RRMS and active SPMS (127). It’s an oral DMT that possesses immunomodulatory and supposed neuroprotective effects involved in reducing oxidative stress without

altering neuronal network activity (128). DMF demonstrated efficacy in reducing clinical relapses and lesion frequencies, together with a tolerable profile, with predominantly mild or moderate gastrointestinal-related adverse effects, such as diarrhoea, nausea, and upper abdominal pain (129,130). While the precise mechanism of action remains unclear, it is believed that the main mechanism of action of DMF involves the direct activation of the nuclear factor erythroid 2–related factor 2 (Nrf2) pathway. Specifically, DMF is a prodrug, and it is rapidly metabolised in its active form Monomethyl fumarate (MMF), which activates the Nrf2 transcription factor by binding to Kelch-like erythroid cell-derived associated protein-1 (KEAP-1). This activation leads to the modification of the transcription of detoxifying and antioxidative genes, resulting in cytoprotective and anti-inflammatory cascade-like effects.

Both DMF and MMF increase the synthesis and recycling of ROS scavenger glutathione and downregulate vascular cell adhesion molecule-1 (VCAM-1) expression in brain endothelial cells, reducing adhesion to activated endothelium and transmigration across the BBB (131–133). Additionally, activation of Nrf2 and direct agonism of MMF of hydroxycarboxylic acid receptor 2 (HCAR2), strongly inhibits nuclear factor kappa-light-chain-enhancer (NF-κB), which is a master regulator transcription factor involved in proinflammatory gene activation. Moreover, by triggering the HCAR2 pathway, DMF has been shown to modulate microglia through the activation of the HCAR2 pathway, reducing neuroinflammation and restoring synaptic alterations in EAE (134). Derived from the Krebs cycle intermediate fumarate, DMF and its metabolite can irreversibly modify and deactivate the catalytic cysteine of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This down-regulates aerobic glycolysis, affecting highly glycolytic effector T cells and activated innate cells, resulting in anti-inflammatory effects (135). Another study showed that DMF succinylates gasdermin D (GSDMD) leading to the formation of S-(2-succinyl)-cysteine, which hinders the interaction of gasdermin D with

caspases, thereby restricting its processing, oligomerization, and its ability to induce cell death. Moreover, in these studies, researchers were able to connect the succinylation of GSDMD amelioration of the clinical score of mice with EAE (136). Moreover, DMF induces hypermethylation of the miR21 region, inhibiting the expression of CCR6 in brain-homing CD4 and CD8 T cells and the polarisation to Th17 cells, resulting in a reduction in the accumulation of autoreactive T cells in the CNS (135).

Administration of DMF to MS patients is associated with an overall increase in the naive T and B cell populations, accompanied by a decrease in the corresponding effector and memory subpopulations (137). Moreover, its use has been linked to an increase in IL-10-producing Bregs (138,139). In vitro studies suggest DMF reduces DC maturation, affecting their capacity to drive Th1 and Th17 cell differentiation (140). Both DMF and MMF inhibit the synthesis of various proinflammatory mediators and induce a shift of macrophages from a proinflammatory M1 phenotype to an anti-inflammatory M2 phenotype (141). In experimental autoimmune encephalomyelitis (EAE) mouse models, commonly used in MS research, DMF improves the preservation of myelin, neurons, and axons (142,143). Thus, DMF exhibits pleiotropic effects on immune cells, glia, and neurons, altering the composition and phenotype of immune cells to confer anti-inflammatory phenotype and exerting antioxidative, neuroprotective and general cytoprotective properties. Recently, in 2019, Diromexil Fumarate was approved for RRMS and active SPMS, which have the same mechanism of action as DMF but improved gastric tolerability profile (144).

In 2013 another monoclonal antibody was approved for the treatment of SPSM and drug-resistant RRMS. Alemtuzumab is a humanised monoclonal antibody targeting CD52, a protein present on the surface of mature leukocytes but not expressed by precursors. The mechanism of action of alemtuzumab in MS

involves the depletion of T and B lymphocytes through direct induction of CD52-mediated apoptosis, and induction of antibody-dependent and complement-mediated cytolysis (145). Specifically, Alemtuzumab induces a rapid depletion of CD52-positive cells, which includes a majority of T and B lymphocytes, and this is followed by a slow and complex process of immune reconstitution. This depletion is followed by a repopulation of lymphocytes, which is thought to result in a reprogramming of the immune system. The repopulation process is slow and can lead to a shift in the ratios of different lymphocyte subtypes, potentially reducing the autoimmune cell response against myelin in MS (146). The reconstitution can lead to a reshuffling of the immune cell repertoire and has been associated with qualitative changes in the immune system, with an increase in functional Tregs, a decrease in self-reactive MBP-specific T cells, and Th17 and Th1 CD4 T-cells (147). Alemtuzumab is highly effective in clinical trials, with a significant reduction in the risk of sustained accumulation of disability and a decrease in the rate of relapse in MS patients compared to other treatments such as IFNβ (140). However, the precise mechanism through which alemtuzumab achieves its therapeutic effects in MS remains unclear. The side effects of alemtuzumab are considerable and can include infusion reactions, infections due to the compromised immune system and B-cell-mediated autoimmune diseases. The most frequently reported side effects include rash, headache, thyroid issues, fever, and infections such as urinary tract infections and upper respiratory infections. More severe side effects can involve immune thrombocytopenia, kidney disorders, and an elevated risk of malignancies, including thyroid cancer, melanoma, and lymphoproliferative disorders (148,149).

In March 2017, the US Food and Drug Administration (FDA) approved ocrelizumab as the first treatment for PPMS and as the first monoclonal antibody for SPMS. Ocrelizumab is a recombinant humanised monoclonal antibody that targets CD20-expressing B cells (150). CD20 is a glycosylated phosphoprotein found on the surface of various B-cells, including pre-B cells,

naïve B cells, and memory B cells. Lymphoid stem cells and plasma cells do not express CD20 and are not directly targeted by ocrelizumab, allowing physiological antibody production to continue. When ocrelizumab binds to CD20-expressing B lymphocytes, it induces antibody-dependent cellular cytolysis and complement-mediated lysis, leading to the depletion of B cells. B cells play a central role in the pathogenesis of MS, including the activation of pro-inflammatory T cells, secretion of pro-inflammatory cytokines, and production of autoantibodies against myelin (150).

Ocrelizumab's effects extend beyond the depletion of B cells, affecting both humoral and cellular immune responses. It also depletes CD20+ T lymphocytes, considered a highly pathogenic immune subset in MS, and over time leads to a reduction in CD8 T cells (151,152). Additionally, a recent study found that anti-CD20 therapy in MS patients is associated with a reduction in circulating Tfh cells through the attenuation of CD27 signalling (153).

Ocrelizumab commonly causes heightened susceptibility to viral infections affecting the skin, sinuses, respiratory tract, stomach, and bowels. Additional adverse effects may involve diarrhoea, irritability, reduced appetite, diminished interest or pleasure, and difficulty concentrating. Serious complications associated with ocrelizumab include PML, hepatitis B reactivation and infusion reactions.

Cladribine, a synthetic purine nucleoside analog, was approved for RRMS in 2017 and for active SPMS in 2019. Originally developed for haematological malignancies, its ability to selectively reduce T and B lymphocytes led to its adaptation as an oral treatment for MS (154). Cladribine is an analog of deoxyadenosine, disrupting DNA synthesis and repair (155). It achieves this by intercalating into DNA and inhibiting enzymes involved in DNA metabolism. Cladribine is an analog of deoxyadenosine (2-chloro-2'-deoxyadenosine) that exhibits partial resistance to adenosine deaminase (ADA). The precise mechanism by which Cladribine exerts its effects is not fully elucidated.

Generally, Cladribine enters cells via specific nucleoside transporters and within the cytoplasm, it undergoes phosphorylation by the enzyme deoxycytidine kinase (DCK), leading to the formation of the mononucleotide 2-chlorodeoxyadenosine 5'-monophosphate (2-CdAMP), which accumulates and integrates into the DNA of cells. This causes an imbalance in triphosphorylated deoxynucleotide pools, inhibiting DNA synthesis and repair, depleting nicotinamide adenine dinucleotide (NAD) and ATP, and ultimately leading to cell death.

Cladribine exerts an immunomodulatory effect on various immune cell populations, influencing their proliferation, maturation, and activation (156). While cladribine primarily targets B and T cell populations, particularly memory B cells, for depletion, it also affects other immune cells such as NK cells and monocytes, albeit to a lesser degree (156). Therefore, cladribine's therapeutic efficacy likely stems from its broad impact on both adaptive and innate immune components. Cladribine is recognized as a semi-selective therapy for immune reconstitution, promoting long-term remission with a brief treatment course. It effectively reduces circulating immune cells, including those implicated in damaging the brain and spinal cord in MS. Even after immune cell counts recover post-treatment, cladribine continues to exhibit beneficial effects, potentially by reducing classical monocyte activation (156). Recent research also suggests it may hinder memory T cell migration across the BBB (157).

This oral therapy is administered in two-week treatment cycles annually: one cycle at the start of the first month and another at the start of the second month during years 1 and 2, with the potential for no further treatment in years 3 and 4. Common side effects include lymphopenia, affecting approximately one in four to one in three treated patients. Other frequent adverse effects comprise upper respiratory infections and headaches. Additionally, there have been reports of malignancies and infections (158).

However, in managing MS, besides disease-modifying approaches, comprehensive care should prioritise symptomatic management to improve overall quality of life. Symptomatic treatments encompass a broad spectrum, including pharmacological interventions for managing spasticity, pain, and fatigue, as well as rehabilitative approaches to address mobility and cognitive impairments. This holistic approach recognizes the diverse and often interrelated symptoms experienced by individuals with MS and emphasises the importance of a multidisciplinary care model.

1.4 Immunosuppression comes at a cost: where do we stand?

Since 1993, when the first immunomodulatory drug for MS was approved, the landscape of available treatments for relapsing and progressive forms of MS has increased significantly, together with the quality of life of the patients carrying this disease. As explained in the previous section, many different classes of drugs are used for the treatment of MS, targeting different immune actors involved in the pathogenesis and perpetration of the disease. Even though these drugs work differently, they are all designed to treat MS by working with the same rationale and concept.

Indeed, most immunosuppressants act by dampening immune responses in an attempt to mitigate or eliminate autoreactivity. This means that while autoreactivity is reduced or avoided, also physiological and protective immunity is affected. Moreover, while these drugs can slow down the progression of the disease and reduce the frequency of relapses, they are not curative and need lifelong administration.

Thus, patients with MS and other autoimmune diseases are obliged to a prolonged state of immunosuppression, which represents an important threat to their health for several reasons.

Firstly, the broad immunosuppressive effects associated with therapy increase susceptibility to infections. Patients receiving such treatment face elevated risks of bacterial, viral, and fungal infections, which can lead to complications and compromise overall health. The types of infections that manifest often reflect the specific immunosuppressive regimen employed and whether there are concurrent immunomodulatory viral infections (159). For example, common infections like pneumonia, cholangitis, and endocarditis are more prevalent among individuals undergoing immunosuppressive therapy (160). Viral infections, particularly herpesviruses such as Cytomegalovirus (CMV), Epstein-Barr Virus (EBV), Herpes Simplex Virus (HSV), as well as John Cunningham virus (JCV) and Human Immunodeficiency Virus (HIV), pose significant risks to those severely immunosuppressed (159). Furthermore, prolonged immunosuppression has been associated with diminished vaccine effectiveness, leaving patients vulnerable to preventable infections (161,162). This concern is particularly relevant in the context of emerging infectious diseases and the necessity for routine vaccinations.

Moreover, prolonged use of immunosuppressive drugs is often associated with adverse effects on vital organs, including the liver, kidneys, and cardiovascular system. Organ toxicity can manifest as nephrotoxicity, hepatotoxicity, and an increased risk of cardiovascular events (163–165). The cumulative impact of long-term exposure to these drugs raises questions about their safety profile over extended treatment durations.

Immunosuppressive therapy, particularly in the context of inflammatory bowel disease (IBD), RA, and SLE has been associated with an increased risk of lymphoma, specifically non-Hodgkin’s lymphoma (166). This is thought to be

due to the weakening of the host immune system's surveillance of tumour cells, allowing for the proliferation of transformed cells. Finally, patients with autoimmune diseases have an increased risk of developing diseases including skin cancer (167). This risk is thought to be increased by chronic immunosuppression from therapies used to manage autoimmune conditions.

In addition to this, discontinuation of immunosuppressive drugs, whether due to side effects or the achievement of a stable disease state, can often lead to disease relapse. Indeed, the abrupt withdrawal of immunosuppression may trigger a resurgence of autoimmune activity. Several studies have reported an increased risk of clinical relapses following the cessation of natalizumab therapy, with some patients experiencing a level of disease activity beyond that observed before initiating the treatment (168–170). Specifically, research has shown that the annualised relapse rates (ARR) can peak at 4 to 6 months after natalizumab discontinuation.

Finally, the use of some immunosuppressants have been linked to abrogate beneficial regulatory networks that are fundamental in limiting autoreactive responses (171).

The dual nature of immunosuppressants—targeting pathogenic autoimmune responses while concurrently depressing the immune system—underscores a delicate balance in their use, which is crucial to achieving therapeutic efficacy in managing autoimmune diseases without compromising the body's ability to mount an effective immune response against external pathogens. This dynamic aspect of immunosuppressant therapy highlights the ongoing efforts in medical research to develop more targeted approaches, seeking to enhance the specificity of treatment while minimising potential side effects related to general immune suppression.

Considering this, the prolonged use of immunosuppressants underscores the complex and chronic nature of autoimmune diseases and the ongoing need for effective, sustained therapeutic interventions.

1.4.1 Antigen-specific therapies for the treatment of autoimmunity

The drawbacks associated with traditional immunosuppressive therapies have paved the way for a paradigm shift towards more specific approaches, able to mitigate the autoreactive responses that underlie autoimmunity without causing generalised depression of immunity, thus avoiding the downsides of conventional drugs and guaranteeing precision targeting and preservation of immune response. Indeed, the goal in treating autoimmune disease would be to identify the specific autoantigens that are targeted by the autoreactive cells and induce tolerance towards those antigens, achieving re-education of the altered immune system. In other words, autoimmune patients need an antigen(s)-specific therapy.

Indeed, moving from the concept of non-specific immunosuppression to the one of antigen-specificity represents a paradigm shift in the ideal treatment of autoimmune diseases, emphasising precision targeting of the immune response. Unlike immunosuppressive drugs that primarily manage symptoms and not the underlying pathogenic process, antigen-specific therapies have the potential to induce immune tolerance, promoting a state of sustained remission toward known specific antigens. By reprogramming the immune system to recognize self-antigens as tolerable, these therapies aim to address one of the root causes of autoimmune diseases, offering the prospect of long-lasting therapeutic benefits. Moreover, antigen-specific therapies can be designed to target specific desired autoantigens implicated in the pathogenesis of

autoimmune diseases, opening the door to highly personalised medicine, where, ideally, treatment approaches could be tailored to the individual's immune autoreactivity profile. These therapies aim to target specifically autoreactive clones and reduce them to tolerance without interfering with physiological immunity, minimising side effects associated with non-specific immunosuppressive drugs. Indeed, by selectively targeting the aberrant immune response, antigen-specific therapies could present a more favourable safety profile compared to traditional immunosuppressive agents.

In addition, while none of the currently approved drugs for MS or other autoimmune diseases are curative nor stop the disease, tolerogenic antigen-specific therapies could theoretically be curative, according to the theory of “infectious tolerance”.

Infectious tolerance, first proposed in the early 1970s (172), is a concept that suggests that the induction of immune tolerance in one subset of cells can extend its regulatory influence to neighboring immune cells, effectively 'infecting' them with a tolerogenic phenotype (173). This phenomenon hints at the potential for sustained tolerance of self-antigens through the initialisation and propagation of a chain of tolerance-inducing signals.

Studies in mice (174) suggest that Tregs are the main actors in infectious tolerance, given their capability to imprint other cell types with tolerogenic features. For example, within skin grafts and DCs, FoxP3+ Tregs have been shown to deplete essential amino acids, leading to inhibition of T-cell proliferation and mTOR signalling and differentiation into Tregs (175).

In general, Foxp3+ Tregs can achieve infectious tolerance by directly transforming conventional T cells into induced iTregs through the secretion of suppressive cytokines such as TGF-β, IL-10, or IL-35 or through interaction with specific subsets of tolDCs (176,177).

Specifically, several studies point out that nTreg- and pTreg-induced tolDCs play an important role in tolerance induction, and more specifically, infectious tolerance. Specifically, DCs autocrine, paracrine, and T cell-derived TGF-β signalling have been shown to initiate the tolerogenic pathway of tryptophan catabolism through the mediation of IDO, causing a surge in regulatory kynurenines, actively contributing to the establishment of a state of infectious tolerance to other immune cells (178).

In addition, tolDCs have been identified as the most potent inducers of Tregs (176). Specifically, tolDC production of IL-10, retinoic acid and TGF-β (171) can trigger self-autonomous tolerogenic feedback loops in which the expansion of Treg and deletion or anergisation of T effector cells is transferred from one immune cell to another. Moreover, in addition to epithelial cells, also thymic DCs have been described to induce central tolerance and more specifically to induce Tregs (179). According to these studies, Tregs can induce tolDCs that are able to induce Tregs, and vice versa. The existence of a bidirectional interaction between Tregs and tolDCs represents the trigger of the sequence of events that lead to infectious tolerance, potentially leading to complete re-education of the autoreactive immune system to tolerance and, ideally to a cure of the disease.

Thus, the potential for antigen-tailored therapies holds promise in optimising treatment outcomes and minimising adverse effects for patients with autoimmunity.

1.5 Human immunogenic and tolerogenic dendritic cells

DCs are specialised APCs that bridge innate and adaptive immune responses. They encompass a diverse range of innate immune cells, including

conventional DCs (cDCs), plasmacytoid DCs (pDCs), and inflammatory DCs (infDCs). DCs recognize various PAMPs and damage-associated molecular patterns (DAMPs) through PRRs like Toll-like receptors (TLRs) (173).

Their primary function involves processing and presenting antigens to T cells, thereby guiding their differentiation depending on the nature of the infection.

iDCs patrol tissues where they maintain tolerance of self-antigens. Indeed, iDCs exhibit a distinctive phenotype characterised by limited cross-presentation capability and reduced expression of co-stimulatory molecules compared to mDCs. iDCs load themselves with self-antigens obtained from apoptotic cells. Exposure to apoptotic cells programs iDCs to become refractory to activation through activation of the Gas6-MERTK pathway (180) and to migrate to lymph nodes to delete or anergise self-reactive T cells.

On the other hand, upon recognition of PAMPs/DAMPs and PRRs, iDCs, which possess high phagocytic activity, internalise microbial proteins, initiate antigen presentation with MHC molecules, and undergo a process of activation and differentiation into mDCs. This maturation involves increased expression of MHC class II, co-stimulatory molecules like CD80 and CD86, cytokines, and the chemokine receptor CCR7, guiding mDCs to secondary lymphoid organs. Within lymph nodes, mDCs present processed antigens to naïve CD8 and CD4 T lymphocytes (181).

However, DCs not only orchestrate immune responses against foreign invaders but also, as previously discussed, play a crucial role in maintaining immune system balance during steady-state conditions and promoting immune tolerance. In this role, DCs are instrumental in eliminating autoreactive cells within the thymus during T lymphocyte development as part of central tolerance mechanisms. Collaborating with medullary thymic epithelial cells, DCs present self-antigens to autoreactive single positive CD4+ T cells via MHC-II, leading to apoptosis through robust interaction (181).

As previously discussed, despite its almost perfect effectiveness, this process does not completely eliminate autoreactive clones. To address this challenge, additional

mechanisms of peripheral tolerance contribute to maintaining immune system equilibrium and preventing reactions against self or harmless antigens. Once again, DCs play a pivotal role in these regulatory mechanisms (174,175).

While iDCs also have tolerogenic features, generally, we refer with the term “tolDCs” to DCs exhibiting a stable semi-mature phenotype and possessing tolerogenic characteristics are recognized as tolDCs. Indeed, iDC can activate and mature, becoming immunogenic, when exposed to PAMPs or DAMPs, while tolDCs maintain their tolerogenic profile even under these circumstances (182,183).

Moreover, tolDCs are characterised by a distinct phenotypic profile in comparison to both iDCs and mDCs, even though they resemble more to iDCs. Unlike their proinflammatory counterparts, tolDCs express low levels of co-stimulatory molecules such as ICOSL and CD80/CD86 (184) and low to intermediate level of HLA-DR expression, making them capable to induce anergy in CD4 and CD8 T-cells due to low signal 1 and 2 (185). Additionally, tolDCs often exhibit an enhanced expression of inhibitory receptors like PD-L1, Immunoglobulin-like transcript 3 and 4 (ILT3 and ILT4), which contribute to the suppression of T cell activation and proliferation (186).

In the context of humans, a distinct subset of DCs known for their tolerogenic characteristics, identified as DC-10, has been observed in peripheral blood in vivo (187). These cells induce reduced responsiveness in allogeneic CD4+ T cells, secrete IL-10, and express surface markers such as CD163, CD141, CD16, and CD14. Furthermore, there is evidence supporting their ability to promote the generation of CD49b+LAG-3+IL10+ Tr1 cells (187). The production of IL-10 theoretically gives these tolDCs the ability to induce anergy

in CD4+ and CD8+ T cells in in vitro models, along with promoting suppressive functions as demonstrated in experimental studies (188,189). Despite sharing surface markers with various types of DCs, including specific subsets of cDCs and infDCs, the exact origin of human DC-10 remains unclear (190).

Additionally, tolDCs have the capability to produce retinoic acid, a vitamin A1 metabolite secreted by specific dendritic cells. This facilitates TGF-β-driven generation of Tregs in vitro and inhibits the differentiation of Th17 cells in the EAE model (191). Certain types of tolDCs also express IDO, an enzyme that metabolises tryptophan into various by-products. Depletion of tryptophan inhibits T cell proliferation and ultimately leads to apoptosis (192).

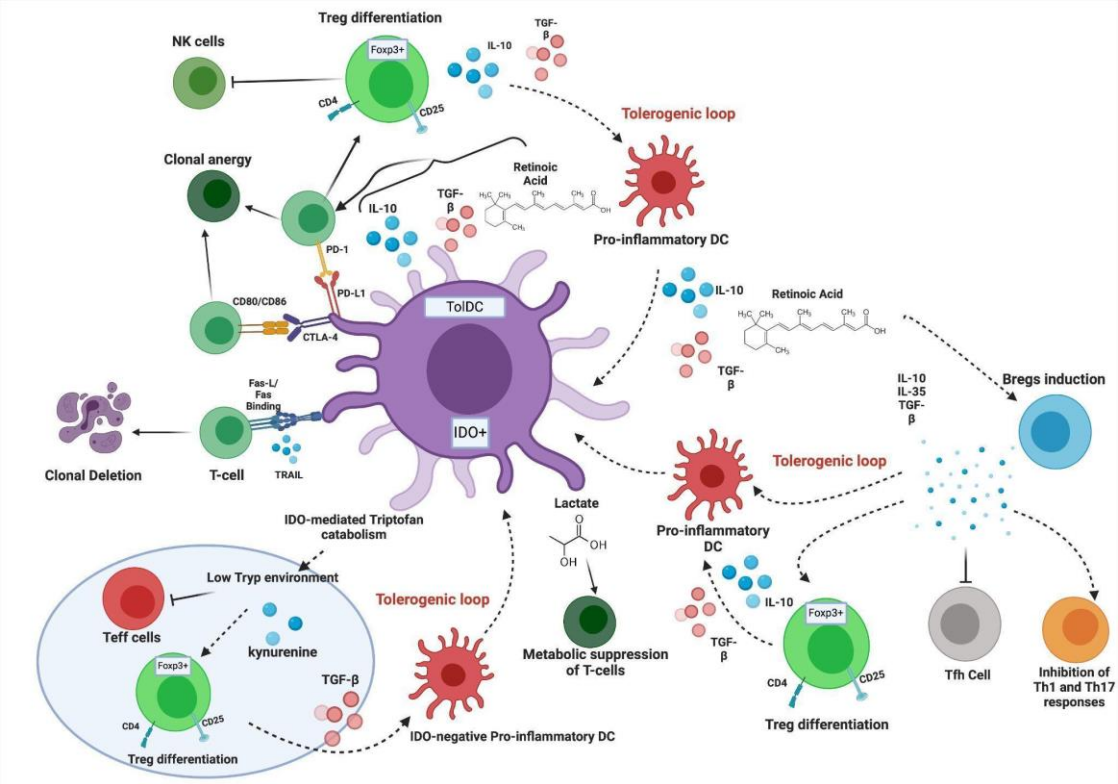


Figure 3: Mechanisms of tolerance exerted by tolDCs (generated with Biorender).

Kynurenine, one of these tryptophan catabolites, is a natural ligand of the aryl hydrocarbon receptor (AHR), promoting Treg differentiation in animal models (193). Furthermore, research indicates that human monocyte-derived (mo-

DCs) tolDCs limit CNS autoimmunity in EAE through metabolic modulation involving lactate secretion (194).

Lastly, tolDCs exhibit the capacity to suppress T cells directly through clonal deletion mechanisms. For example, the interaction between tumor necrosis factor-related apoptosis-inducing ligands (TRAIL) on DCs and death receptors on T cells can trigger apoptosis through triggering of the caspase pathway (195). Finally, Fas, which is upregulated in activated CD4 T cells, can engage with Fas ligand (FasL) present on tolDCs, leading to apoptosis of T cells (196).

1.5.1 Transcriptional drivers of dendritic cells

The differentiation of innate immune cells, and specifically the acquisition of an immunogenic or tolerogenic function of DCs, is intricately regulated by transcription factors and epigenetic mechanisms. The pathways that guide progenitor cells into specific DC subsets and their development into either immunogenic or tolerogenic states are not completely understood. However, several key transcription factors have been identified through research in both mice and humans.

In mice, PU.1 is essential for the development of cDCs, as it activates DC-SCRIPT to promote cDC differentiation over pDCs. The transcription factor E2-2 is crucial for maintaining pDC identity, and its absence leads to the transformation of pDCs into cDC-like cells. Other transcription factors such as ID2, IRF4, IRF8, BATF3, NFIL3, NOTCH2, and KLF4 play significant roles in the polarisation of DC subsets (190). Additionally, AHR is critical in steering progenitor cells towards the DC program instead of the macrophage lineage. In humans, AHR activation promotes the differentiation of mo-DCs through the transcription factor BLIMP-1 (197).

Even though immature DCs can be considered tolerogenic, once that maturation is triggered DCs can still technically develop into either tolerogenic or immunogenic phenotypes. In an in vitro model using murine bone marrow progenitors to study DC differentiation, it was found that IRF4 is critical for the development of tolerogenic properties, as for the capability to induce Th2 responses (198). Importantly, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) has been shown to influence monocytes to acquire suppressor functions through the triggering of the IFN-γR/IRF-1 and AKT/mTOR pathways (199).

On the other hand, NF-κB is indispensable for immunogenic differentiation, as it enhances the production of proinflammatory cytokines and the expression of antigen presentation molecules (200). However, this picture is complicated by the fact that NF-κB signalling is also vital for DCs to survive and to sustain immune homeostasis (201).

Another important factor is nuclear receptor corepressor 1 (NCoR1). which suppresses tolerogenic responses in murine DCs (194). The absence of NCoR1 can cause increased expression of immunomodulatory genes and a higher proportion of regulatory T cells (Tregs). On a mechanistic level, NCoR1 downregulates PU.1-bound enhancers on tolerogenic genes and promotes the translocation of the NF-κB family member RelA to the immune cell nucleus (190). Moreover, when NCoR1 is conditionally knocked out in human hematopoietic stem cells, there is a rise in Treg numbers and a delay in leukaemia development in mouse models (190).

Several transcription factors collaborate with epigenetic enzymes to target specific genomic regions, resulting in significant epigenetic changes. DNA methylation and modifications of histones play crucial roles in defining cellular phenotype and function. For instance, histone deacetylase 11 (HDAC11) suppresses IL10 expression in both human and mouse dendritic cells (202,203). Furthermore, oxidised phospholipids can induce a tolerogenic

phenotype in human monocyte-derived dendritic cells (mo-DCs) by inhibiting histone H3 serine 10 phosphorylation (H3S10ph), thereby reducing their capacity to activate T cells. DNA methylation also plays a critical role in tolerogenesis: for example, prostaglandin E2 (PGE2) triggers the upregulation of DNMT3A in human monocytes, leading to hypermethylation and silencing of proinflammatory genes (204).

Although the study of histone modifications and DNA methylation in DC tolerogenesis is limited, current evidence underscores the significance of epigenetic modifications in immune cell differentiation.

For instance, STAT6-induced DNA methylation changes are pivotal for defining the DC phenotype as opposed to macrophages (205). Additionally, vitamin D3 triggers DNA demethylation and gene expression changes in tolDCs (206). This process involves the vitamin D receptor directly recruiting TET2, an enzyme essential for active DNA demethylation, and autocrine IL-6 production.

Our comprehension of the epigenetic mechanisms underlying DC tolerogenesis is still developing, yet investigating the modulation of epigenetic enzymes to optimise tolDC properties for therapeutic applications remains a promising area of research.

1.5.2 In vitro generation of tolDCs

Because iDCs inherently exhibit phenotypic instability, transitioning from tolerogenic to immunogenic states under inflammatory conditions, and due to the challenges in isolating rare human DC subpopulations with tolerogenic characteristics, several methodologies have been developed to generate tolerogenic DCs (tolDCs) from human monocytes in vitro, often referred to as mo-tolDCs.

Typically, tolDCs can be generated in vitro by starting with the isolation of peripheral CD14+ monocytes, which are then cultured with IL-4 and GM-CSF to induce DC differentiation. Simultaneously, a "tolerizing" protocol is applied to induce semi-mature tolDCs, which involves various approaches utilising a wide array of molecules and treatments.

For example, tolDCs can be efficiently generated by adding IL-10 to the culture conditions (207,208) or by transfecting monocytes with the IL-10 gene to produce high quantities of this cytokine (209). These cells produced in vitro in the presence of IL-10, resemble naturally occurring IL-10-producing human tolDCs, with a high capability to induce Tr1 cells through the ILT4/HLA-G pathway (210).

TGFβ is also able to induce tolDCs both in vivo as in vitro protocols, alone or in combination with other molecules (211,212). Antibiotics and Glucocorticoids, like minocycline and dexamethasone, are also able to imprint mo-DCs with a tolerogenic phenotype (213,214) able to produce IL-10 and TGFb, inhibit T-cell proliferation and proinflammatory cytokine production, as inducing a T-helper 2 (Th2) signature.

TolDC can also be generated with Rapamycin, which functions by inhibiting the mechanistic target of rapamycin (mTOR), a key regulator of the immune system. Rapa-treated tolDC can induce Tregs and T-cell apoptosis (207). Specifically, these tolDCs can induce apoptosis of alloreactive CD4+ T cells dependent on IFNγ, resulting from the elevated production of IFNγ by T cells interacting with them (215). However, despite that they have a more mature phenotype in comparison with other types of tolDCs, Rapamycin-treated tolDCs induce low proliferation of allogeneic T cells, similar to other tolDCs (215,216).

Finally, other groups generate tolDC by specifically knocking out or overexpressing target genes during the differentiation from monocytes to DCs. In this sense, a plethora of approaches and targets have been investigated. For example, some researchers generated tolDCs by downregulating the expression of costimulatory molecules as CD40, CD80 and CD86 (217), overexpressing IL-10 (as previously mentioned) (209) or IL-23-suppressor factor and IL-12 (186). In general, all in vitro mo-derived tolDC share properties encompassing high immunosuppressive cytokine production, low expression of costimulatory proteins, and the ability to inhibit T cell proliferation and activation.

The set-up of various protocols for generating tolDCs, along with a deeper understanding of how they induce tolerance, has fueled research into their potential use in treating autoimmune diseases and inflammatory disorders like RA and MS. Several ongoing clinical trials are currently evaluating the efficacy, safety, and tolerability of tolDCs for these conditions. Therefore, it is essential to thoroughly understand the mechanisms by which tolerogenic dendritic cells promote tolerance, allowing for a comprehensive assessment of the characteristics and potential clinical benefits of different types of mo-tolDCs.

1.5.3 Clinical Trials involving tolerogenic dendritic cells

The use of tolDCs in autoimmunity and transplantation theoretically allow the reestablishment of tolerance of autoantigens without unwanted immunosuppression. This concept, together with the expanding knowledge of tolDC biology and functionality through *in vitro* (218–220) and *in vivo* studies (221–226) and the optimisation of different protocols for their generation in

laboratories eventually led to the idea to implement tolDCs as cell therapies for the treatment of autoimmunity and in transplantation, fueling the initiation of several clinical trials. Several phase I clinical trials explored the use of different types of tolDCs for the treatment of various conditions, as resumed in Table 1.

Table 1. Clinical trials involving tolDCs in autoimmunity

ID	Phase	Design	Status	Indication	Cell type	Route	Administration scheme
Autoimmune diseases							
NCT00445913[i]	I	Randomized, single group assignment, double-blind	Completed	T1D	BM-derived DCs treated with antisense oligonucleotides targeting CD80, CD86, CD40	i.d	4 Injections, bi-weekly
NTR5542[ii]	I	Nonrandomized, single arm	Completed	T1D	Proinsuline-loaded VitD3-tolDCs	i.d.	2 Injections with 28-day interval
NCT02354911[iii]	II	Randomized, double blind, placebo-controlled, cross-over study	Unknown	T1D	BM-derived DCs treated with antisense oligonucleotides targeting CD80, CD86, CD40	i.d.	4 Injections, bi-weekly
NCT03895996[iv]	I/II	Randomized, parallel assignment, placebo controlled, double-blind	Recruiting	T1D	Autologous dendritic cell therapy (AVT001)	i.v.	3 Monthly injections
NCT03337165[v] (TolDCfoRA)	I	Single group assignment, open label	Completed	RA	Dex-tolDCs	i.a.	Dose escalation, single injection
Rheumavax	I	Nonrandomized, control group, open label	Completed	RA	NF-κB inhibitor-treated DCs, loaded with citrullinated peptides	i.d.	2 Progressive dose levels
CRiSKCT0000035[vi] (CreaVax-RA)	I	Interventional, single arm, open label	Completed	RA	DCs pulsed with PAD4, HNRNPA2B1, citrullinated filaggrin, and vimentin antigens		5 Injections according to two dose regimens: low and high
NCT01352858[vii] (AutoDECRA)	I	Randomized, parallel assignment, open Label	Completed	RA	Dex/VitD3-tolDC loaded with autologous synovial fluid	i.l.	Dose escalation, single injection
NCT02283671[viii]	I	Single group assignment, open Label	Completed	MS, neuromyelitis-optica	Dex-tolDCs loaded with myelin peptides or aquaporine-4-derived peptide	i.v.	Dose escalation, 3 injections administered bi-weekly
NCT02903537[ix] (TOLERVIT-MS)	I/IIa	Nonrandomized, parallel assignment, open label	Recruiting	MS	VitD3-tolDCs loaded with a pool of myelin peptides	i.n.	Dose escalation, 6 injections: 4 bi-weekly and 2 monthly
NCT02618902[x]	I/IIa	Nonrandomized, parallel assignment, open label	Recruiting	MS	VitD3-tolDCs loaded with a pool of myelin peptides	i.d.	Dose escalation, 6 injections: 4 bi-weekly and 2 monthly
2007-003469-42[xi]	I	Sequential-cohorts, dose-range	Completed	Crohn's disease	Dex/VitA tolDCs	i.p.	Dose escalating, single injection vs 3 injections bi-weekly
NCT02622763[xii]	I	Randomized, parallel assignment, single blind	Terminated (low recruitment)	Crohn's disease	Dex-tolDCs	i.l.	Unknown

Table Glossary: BM-derived: Bone Marrow-derived; T1D: Type 1 Diabetes; RA: Rheumatoid Arthritis; VitD3: Vitamin D3; Dex: Dexamethasone; DASQ: Disease activity score for RA, calculated on the basis of the tenderness or swelling upon touching of 28 joints; HAQ: The Health Assessment Questionnaire, composed of 20 questions about the ability to perform simple actions and used as a measure to evaluate the outcome of patients with rheumatic diseases; MS: Multiple Sclerosis; i.v: intravenous; i.p.: intraperitoneal; i.d.:intradermal; i.n.:intranodal; i.l: intralesional. **Table modified from** (190).

Results from Phase I clinical trials have been encouraging, showing the safety and feasibility of most cellular products and motivating the birth of Phase II clinical trials looking for proof of efficacy in humans. Three phase I trials have been performed involving patients with MS.

The first clinical trial involved eight individuals with either RRMS or progressive MS and four with neuromyelitis optica spectrum disorders (NMOSD). Specifically, patients received intravenous injections of dexamethasone-induced tolDCs loaded with a combination of seven myelin peptides and an aquaporin-4 peptide (AQ463-76). These injections were administered three times over a period of two weeks in an escalating dose scheme (50, 150, and 300 million tolDCs). The treatment demonstrated clinical safety and was well-tolerated, indicating potential immunoregulatory effects, such as enhanced IL-10 production and reduced numbers of memory CD8+ T cells and NK cells. However, there were technical challenges in administering the highest dose (300 million tolDCs) (227).

Two harmonised phase I trials for active MS patients utilised intradermal (NCT02618902) and intranodal (TOLERVIT-MS, NCT02903537) injections of 5, 10, and 15 million VitD3-tolDCs loaded with seven myelin peptides. Participants received a total of six doses, with the first four administered biweekly and the last two every four weeks (227).

Preliminary findings indicate that both intradermal and intravenous delivery methods for myelin-specific VitD3-tolDCs are safe, feasible, and well-tolerated by patients.

A phase II trial is now enrolling MS patients who will receive either placebo or myelin-specific dexamethasone-induced tolDCs via intravenous infusion every two weeks together with immunomodulatory drugs (TolDecCOMBINEM, NCT04530318) (227). This trial marks the first instance of a combined therapy approach that pairs such immunomodulatory drugs with the goal of enhancing treatment efficacy while mitigating the severe side effects often associated with high-efficacy treatments.

Despite the encouraging outcomes observed, the lack of uniformity in protocols for generating tolDCs—spanning various methods, dosages, administration routes, targeted diseases, and patient groups—has hindered direct comparison of trial results. This underscores the critical need to standardise future clinical trials. Notably, the existence of diverse protocols for tolDC generation, each resulting in distinct phenotypic and tolerogenic profiles, presents a substantial challenge.

Addressing this requires establishing strong quality control criteria to determine the phenotype, efficacy, and safety of each cell product (190).

To enhance reproducibility and standardise both preclinical and clinical research on tolerogenic dendritic cells, a minimum information model for tolAPC (MITAP) was introduced (228). However, subsequent research concluded that MITAP has achieved only partial success, highlighting the necessity for further efforts to standardise tolDC therapies. In addition to MITAP, consortia aimed at harmonising clinical trial design and immunomonitoring protocols involving tolDCs have emerged in both the United States of America (Immuno Tolerance Network) (229) and Europe (Action to Focus and Accelerate Cell-Based Tolerance-Inducing Therapies) (229–231).

Importantly, there is still a lack of conclusive data demonstrating clinical improvement and the induction of tolerance of known autoantigens.

This uncertainty may stem from factors such as the limited size of existing trial cohorts, a current lack of information regarding the optimal administration route and doses, challenges in definitively identifying biomarkers for tolerance induction, and the production of functional tolDCs with low in vivo potency. A critical aspect contributing to this is the transient nature of these cells, as they might have a limited capacity of persisting or expanding in the host.

Therefore, tolDCs must efficiently carry out their tolerance-inducing function within a brief lifespan, playing a crucial role in triggering the long arm of tolerance, represented by regulatory T-cells.

Another element of crucial importance consists in the the migratory capacity of tolDCs to the lymph nodes or to target inflamed tissue where the effector autoimmune response is going on. While tolDCs generally express CCR7 and might need to migrate to lymph nodes to exert their regulatory function (232,233), knowledge regarding the expression of other chemokine receptors that would equip them with migratory capacity to the inflamed brain, joints or other organs is not well known.

The route of administration of tolDCs is indeed directly connected to their migratory capability. Several routes of administration of different tolDC products have been studied in clinical trials, involving intravenous, subcutaneous, intraperitoneal, intradermal, intranodal, and intra-articular routes (227). To complicate the scenario, the different trials were performed using different doses and number of administrations.

Research in non-human primates suggests that intravenous delivery is the most tolerogenic method for administering tolDCs (234). It is also the preferred route when direct access to the target tissue is challenging. However, intravenous administration may require a higher cell count to ensure effective migration to secondary lymphoid organs and target inflamed organs. This is particularly relevant for MS, where the CNS is the target and cells need to cross the BBB. Despite this, the TolDec-EM-NMO trial (NCT02283671) encountered difficulties delivering the highest tolDC dose (300 million cells) intravenously, suggesting that this approach also has practical limitations.

To address these challenges, tolDCs have often been injected directly into or near the target tissue in various clinical trials. For instance, RA studies, TolDCfoRA (NCT03337165) and AutoDeCRA (NCT01352858) used intra-articular injections, while MS-tolDC delivered cells intradermally (i.d.) near cervical lymph nodes. In T1D, intradermal injections near the pancreas were employed. However, due to the limited migration capability of tolDCs with routes like intradermal, subcutaneous, or intraperitoneal and direct intranodal injections have been suggested.

Indeed, in the TOLERVIT-MS trial, myelin-VitD3-tolDCs were successfully administered to cervical lymph nodes in MS patients, even though this method required specialised echography-guided techniques.

Finally, the lack of conclusive evidence regarding in vivo tolDC mechanisms points out the necessity for extensive immunomonitoring in clinical trials to identify potential biomarkers associated with tolerogenicity. In this sense, technological approaches which can detect with high profundity the phenotype of the immune system landscape, like single-cell technologies, represent an important possibility to identify tolerance induction biomarkers.

1.5.4 Vitamin D3 Tolerogenic Dendritic cells

tolDCs can be derived from peripheral monocytes in the presence of Vitamin D3, also known as cholecalciferol. Exploration into the role of Vitamin D3 in DC function began with early observations suggesting its involvement in immune regulation (235), alongside its recognized functions in calcium and bone metabolism. These systemic immunological effects include the suppression of Th1 and Th17 responses, enhancement of Th2 responses in CD4 T-cells, and inhibition of B-cell proliferation (236–238), among others.

Initial investigations into Vitamin D's immunomodulatory properties on the adaptive immune system prompted further exploration of its specific impact on DCs. Pioneering studies by Luciano Adorini and colleagues in the early 2000s (235,239) demonstrated that Vitamin D3 can induce tolerogenic properties in DCs, influencing their capacity to promote immune tolerance in autoimmune disease contexts (240).

Subsequent experiments elucidated the molecular mechanisms underlying this phenomenon. Similarly to other tolerising agents, Vitamin D3 promotes the differentiation of DCs into a tolerogenic phenotype characterised by reduced expression of co-stimulatory molecules, lower HLA-DR expression and an enhanced ability to induce Tregs.

These studies shed light on the intricate relationship between Vitamin D3 and DCs but also hinted at potential therapeutic applications in immune-mediated disorders.

VitD3-tolDCs exhibit low expression of costimulatory molecules, reduced HLA-DR and CCR7 in comparison to mDCs, high expression of CD14 and produce high IL-10 and low IL-12 and IL-6 (185). Moreover, this phenotype is maintained after TLR stimulation with LPS, confirming the stability of the VitD3-tolDC functional profile.

This finding is of particular importance for their application as a cell therapy, given that it suggests that these cells would maintain their tolerogenic profile even in an inflammatory context, as the ones of autoimmune patients (185).

In general, activation of the IL-6-JAK-STAT3 pathway in monocyte-to-DC in vitro cultures induces the differentiation of the DC with tolerogenic properties. Specifically, research from our group (206) showed a novel mechanism whereby Vitamin D stimulation induces JAK2-mediated phosphorylation of STAT3, thereby initiating the differentiation process towards tolDCs.

Notably, the interaction between the vitamin D receptor (VDR) and phosphorylated STAT3 orchestrates the formation of a complex involving methylcytosine dioxygenase TET2, further delineating the molecular cascade underlying the generation of tolDCs. Indeed, pharmacological inhibition of JAK2 reverses the tolerogenic characteristics induced by vitamin D in VitD3-tolDCs.

In addition, previous studies from our group aimed at the identification of a unique biomarker of VitD3-tolDCs identified MAP7 and MUCL1 as genes expressed by VitD3-tolDCs and not by mDCs, iDCs and other types of tolDCs (tolDCs differentiated with rapamycin and with dexamethasone) (241).

Finally, as previously reported, our group has developed an autologous tolDC-based cell therapy produced from peripheral blood CD14+ monocytes differentiated in the presence of the Vitamin D3 (VitD3-tolDCs), which has been administered in two Phase I harmonised, dose-escalating clinical trials including active RRMS patients (NCT02903537 and NCT02618902), showing safety and feasibility.

1.6 Impact of MS inflammation on the innate immune system

Innate immune cells, particularly monocytes, exhibit significant alterations in various autoimmune diseases, including SLE, T1D, and IBD (242–246). This phenomenon is similarly observed in MS, where there are significant alterations in the proportions and absolute counts of monocyte populations, along with changes in their transcriptome, epigenome, metabolism, and overall function (247–250). These transformations are likely influenced by the chronic inflammatory environment typical of such diseases.

When considering the development of therapies based on mo-tolDCs, it is important to recognize that monocytes sourced from patients may exhibit distinct phenotypic differences compared to those from healthy individuals. For instance, monocytes from MS patients, which have been primed by an inflammatory environment, might demonstrate resistance to protocols designed to induce tolerance. This could result in the production of tolDCs that are less effective, thereby diminishing the therapeutic potential of mo-tolDC-based treatments.

Supporting this hypothesis, several studies have documented significant transcriptional, epigenetic, and functional disparities in tolDCs derived from MS patients when compared to those generated from healthy donors (241,251,252). A critical component in the plasticity of myeloid cells is DNA methylation, an epigenetic modification that plays a pivotal role. Enzymes that regulate DNA methylation, such as DNMT3A and TET2, are closely linked with myeloid cell differentiation, functionality, and transcription factor activity (253), thereby influencing cell phenotypes. Moreover, alterations in DNA methylation have been strongly associated with inflammatory responses and autoimmune conditions like MS (254–256).

In addition, our group described how tolDCs induced by Vitamin D3 in MS patients exhibit a different transcriptional profile in comparison to the ones differentiated from healthy individuals (241,257). Moreover, these transcriptional differences are accompanied by a reduction in the production of TGF-β and reduced suppressive capacity of allogeneic proliferation in mixed lymphocyte reaction (MLR).

Additionally, it is important to point out that the close relationship between metabolism and immune cells suggests that metabolic abnormalities might also influence the efficacy of tolDC treatments. In a study of tolDCs derived from a T1D cohort, patients with poor glycemic control produced tolDCs that were less

effective at inducing functional regulatory T cells and inhibiting Th1/Th17 responses compared to those from well-controlled patients and healthy donor (190).

1.6.1 Toward second-generation tolDC therapies

The profound impact of inflammation on the starting material used to create myeloid regulatory cell therapies significantly influences the development of mo-tolDC-based treatments. However, understanding the intricate interplay among transcriptional regulators, epigenetic modifications, and the functional properties of monocytes and DCs in disease underscores the complexity of devising effective therapies for autoimmune diseases.

Successful therapy design requires comprehensive characterization of the inflammatory, transcriptomic, epigenomic, and metabolic immune landscapes specific to each disease. Additionally, detailed phenotypic analysis is essential to discern differences between monocytes and tolDCs derived from healthy individuals versus those affected by autoimmune conditions. This approach is crucial for uncovering the mechanisms underlying treatment failures or suboptimal responses during tolerance induction.

Furthermore, integrating mo-tolDC therapies with other immunomodulatory strategies aimed at reducing inflammation may be necessary to achieve optimal therapeutic outcomes. Indeed, by mitigating inflammation and promoting a regulatory profile in monocytes

Targeting MS-related subsets of immune cells could enhance tolDC-based treatments. In particular, novel approaches are needed to enhance the potency of current tolDC products. Unlike immune cell therapies such as CAR-T cells,

tolDCs may not persist or expand in the host organism, highlighting the critical importance of potency in designing successful therapies.

2. Hypothesis

Evidence supports that the function of innate immune cells is highly dependent on immune niche cues, which act through different mechanisms, involving surface receptors, cell signalling cascades, transcription factors and the epigenetic machinery, which result in changes in epigenome, closely associated with gene expression. Also, it is well established that autoimmune-related inflammation has effects on the phenotype and function of these cells, highlighting the need for studies on myeloid cells isolated from autoimmune patients. Specifically, the alterations that chronic inflammation can induce in innate immune cells could impact the efficiency of tolDC-based therapies.

We hypothesise that:

1) The systemic inflammatory environment generated in the context of MS has an impact on the phenotype and functionality of peripheral blood monocytes, which represent the starting material of our VitD3-tolDC cell therapy.

2) By integrating multi-omic approaches, we can identify dysregulated pathways in monocytes and monocyte-derived mDCs and tolDCs from MS patients in comparison to healthy individuals.

3) By modulating dysregulated pathways in cells derived from MS patients, we can potentially restore fully functional tolDCs for therapeutic purposes.

3. Objectives

In this thesis, our primary objective is to investigate the alterations at the immunological, epigenomic, and transcriptomic levels that occur in peripheral blood monocytes isolated from MS patients and in the tolDCs derived from them and used as cell therapy. This understanding may lead to the development of new strategies to enhance clinical protocols. To accomplish this general goal, we propose the following specific aims:

1) To perform a comprehensive characterization of CD14+ cells isolated from MS patients, and compare them with healthy individuals, at the surface protein, transcriptomic and epigenomic levels.

2) To define whether the potential alterations of the phenotypic profiles of MS-isolated monocytes are maintained following in vitro differentiation into mDCs and tolDCs, by comparing the transcriptomic and epigenomic signature of MS mDCs/tolDCs versus HD mDCs/tolDCs. This could lead to the identification of common alterations in MS across these cell types.

3) To use the information of signalling pathways and factors generated through the omics analysis of the different cell types to modulate identified MS-specific dysregulated pathways to boost the functionality of therapies based on tolDCs derived from MS patients.

4. Materials and Methods

4.1 Patients and Donors

Whole blood samples healthy donors (HD) and RRMS patients were collected by standard venipuncture in lithium heparin tubes (BD Vacutainer® Heparin Tubes) to obtain material to be used for RNAseq, DNA methylation, qPCR and flow cytometry experiments involving primary mixed lymphocyte reaction comparing MS cells to HD ones. Only patients with RRMS and during an active phase of the disease were considered. Patients did not receive any corticosteroids in the month before the blood extraction or any disease-modifying therapy during at least the previous 12 months. In the experiments involving the drug dimethyl fumarate (DMF), RRMS patients treated for more than 6 months with DMF were included. For DNA methylation and RNAseq HD and MS patients were age and sex matched.

To conduct in vitro functional validation experiments and qPCR analyses using Aryl Hydrocarbon Receptor agonist (FICZ), antagonist (CH223191), and Dimethyl Fumarate (DMF), buffy coats from anonymous donors were sourced. The buffy coats used in this study were sourced from the Banc de Sang i Teixits (Barcelona, Spain), following institutional Standard Operating Procedures for blood donation in accordance with the principles set forth in the World Medical Association (WMA) Declaration of Helsinki. Prior to donation, all donors provided signed informed consent.

4.2 Monocyte isolation

MS patient and HD whole blood and buffy coat samples were processed by first enriching the CD14+ fraction using the RosetteSep® Human Monocyte Enrichment Kit (StemCell Technologies, Vancouver, Canada). This was followed by a density gradient separation using Ficoll-Hypaque (Rafer, Zaragoza, Spain). Positive selection of CD14+ cells was then performed using the EasySep® Human CD14 Positive Selection Kit (StemCell), according to the manufacturer's instructions. Cell viability was assessed using 7-amino-actinomycin D (7-AAD) (BD Biosciences, Franklin Lakes, NJ, USA) and phycoerythrin (PE)-conjugated annexin V (Immunotools, Friesoythe, Germany) staining for 20 minutes at 4°C, protected from light. Cell counts were simultaneously quantified using PerfectCount beads (Cytognos, Salamanca, Spain) and Trypan Blue staining (Gibco™). Samples were analysed on a FACSCanto II flow cytometer (BD Biosciences), and monocyte purity was determined using forward and side scatter gating strategies on FACSDiva software (BD Biosciences). If monocyte purity exceeded 90%, the isolated CD14+ fraction was used for downstream applications (RNAseq, DNA methylation arrays) and/or to differentiate mDCs or tolDCs, provided the cell number was sufficient.

4.3 tolDCs and mDCs differentiation

37°C with a density of 1 × 10^6 cells/ml in IMDM culture medium (Gibco™, Thermo Fisher Scientific). The culture medium included 400 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 500 U/ml IL-4 (both from Peprotech, London, UK), supplemented with 2% L-Glutamine (Sigma-Aldrich),

2% Human serum (Sigma-Aldrich), and 25 mM HEPES (Gibco™, Thermo Fisher Scientific). The entire medium and cytokines were refreshed on day 4. To generate mature DCs (mDCs), a maturation cocktail containing 1,000 U/ml IL-1β (Peprotech), 1,000 U/ml TNF-α (Peprotech), and 1 µM prostaglandin E2 (Pfizer, New York, USA) was added on day 4. For the differentiation of tolerogenic DCs (tolDCs), in addition to the maturation cocktail, 1 nM vitamin D3 (Calcijex, Abbott, Chicago, IL, USA) was added on days 0 and 4 to induce vitD3-tolDC differentiation. Experiments involving modulation of the Aryl hydrocarbon receptor (AhR) included the addition of 18 µM 6-Formylindolo [3,2-b] carbazole (FICZ) AhR Agonist (Invivogen) or 30 µM 2-methyl-2H-pyrazole-3-carboxylic acid (CH223191) AhR Antagonist (Invivogen) on days 0 and 4 during differentiation. Experiments with dimethyl fumarate (DMF) included the addition of 10 µM dimethyl fumarate (Sigma) on days 0 and 4 during differentiation. On day 6, cells were harvested, washed twice, and underwent viability quality control using 7-amino-actinomycin D (7-AAD) (BD Biosciences, Franklin Lakes, NJ, USA) and phycoerythrin (PE)-conjugated annexin V (Immunotools, Friesoythe, Germany) before being used for downstream applications.

4.4 Flow Cytometry analysis of monocytes and DCs surface marker expression

Surface expression of CD11c, CD14, CD83, CD86, CCR7 and HLA-DR protein markers in mDC and different types of tolDC from HD or MS patients (w/o FICZ, CH223191 or DMF) was analyzed by flow cytometry. Cell suspensions were incubated for 20 min, protected from light, with the appropriate amounts of monoclonal antibodies anti-CD11c PE-Cy7 (BD Biosciences), CD14 V450 (BD Biosciences), CD83 APC (Biolegend), CD86 FITC (Biolegend), CCR7 PE (Biolegend) and HLA-DR V500 (BD Biosciences). Subsequently, at least 5000

CD11c+ cells for each cell condition were acquired using a FACSCanto II flow cytometer and analysed using FACSDiva software. Analysis of percentages of monocytes subsets in HD, MS and MS DMF patients were performed incubating for 30 minutes 3 million peripheral blood mononuclear cells (PBMCs) with anti-CD14 Spark Blue™ 550 (BioLegend), CD16 PE-AF700 (BioLegend), anti-CX3CR1 Brilliant Violet 711™ (BioLegend), anti-PD-L1 Brilliant Violet 785™ (BioLegend) antibodies and acquiring the samples on a Cytek Aurora Spectral Flower cytometer and analysed in OMIQ software.

4.5 Mixed Lymphocyte Reaction suppression assays

To isolate allogeneic PBMC, whole blood samples of healthy individuals were processed by ficoll-hypaque density gradient separation. Then, PBCMs were stained with BD Horizon™ Violet Proliferation Dye 450 (BD) DNA dye and co-cultured in 96-well round bottom plates, in a total volume of 200 µl of supplemented RPMI medium, at a ratio of 1:20 with either MS-derived or HD-derived mDC, tolDC, tolDC FICZ, tolDC CH or tolDC DMF, according to the experiment. Cells were kept for 4 days at 37°C in a 5% CO2 atmosphere, and then the V450 positive fraction was calculated for each condition by using a FACS BD Lyrics flow cytometer. Negative controls comprehending PBMCs-only and positive controls with 50 ng/mL phorbol 12-myristate-13-acetate (PMA) and 500 ng/mL ionomycin (Thermo Fisher Scientific) were also used. Percentages of cell proliferating in the different tolDC conditions were then normalized to the percentage of proliferation induced by mDC, used as controls, and multiplied by 100, obtaining the percentage a suppression of proliferation. When comparing HD and MS patients, samples were matched by sex and age.

4.6 Mixed Lymphocyte Reaction T cell polarisation assay

PBMCs were isolated from sex-matched healthy donors' buffy coats as for MLR Suppression assays. Then, PBCMs were co-cultured in 96-well round bottom plates, in a total volume of 200 µl of supplemented RPMI medium, at a ratio of 1:2 with different types of HD DCs (HD mDCs, HD tolDCs, HD tolDCs + DMF, HD tolDCs DMF or HD tolDCs DMF + DMF) and in presence/absence of 10 uM DMF during the differentiation to tolDCs or during the coculture. Negative controls comprehending PBMCs-only were also used. Cells were kept for 6 days at 37°C in a 5% CO2 atmosphere, stained with a panel of antibodies comprehending anti-CD3 V450 (BD Biosciences), anti-CD4 PerCPCy5.5 (BD Biosciences), anti-CD45RA Pe-Cy7 (BD Biosciences), anti-CCR7 PE (Biolegend), anti-CCR6 and v-CXCR3 AF488 (Biolegend) and then acquired in a LSR Fortessa flow cytometer (BD Biosciences).

4.7 DNA and RNA extraction

DNA from monocytes, mDC and different types of tolDC from HD and MS patients was extracted with a DNeasy Blood & Tissue Kits (Qiagen) following manufacturer’s instructions, while total RNA was isolated by using RNeasy Blood & Tissue Kits kit (Qiagen) following manufacturer’s instructions. DNA and RNA was quantified with a Nanodrop ND-1000 spectrophotometer (ThermoFisher Scientific) and used for downstream applications.

4.8 Retrotranscription and qPCR

Total RNA was retrotranscribed into cDNA by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). cDNA

was then used to perform a quantitative RT-qPCR reaction prepared with LightCycler480 SYBR Green I Master (Roche) and analysed with a LightCycler 480 instrument (Roche). Primers used in this analysis were designed with Primer3 software (258) or bought from commercial vendors (ThermoFisher Scientific). B2M was used as a housekeeping gene and ΔΔCt method was used to analyse the relative quantities of genes of interest.

4.9 Bisulfite conversion and DNA methylation analysis

500 ng of genomic DNA was bisulfite-converted using the EZ DNA Methylation-Gold kit (Zymo Research) according to the manufacturer's instructions and hybridized on Infinium MethylationEPIC BeadChip arrays (Illumina, Inc., San Diego, CA, USA). This technology allows analysis of over 850,000 methylation sites per sample at single nucleotide resolution, covering 99% of RefSeq genes and 95% of CpG islands. Image processing and intensity data extraction were performed as previously described (259). Each methylation data point consisted of the combined fluorescent intensities of Cy3 and Cy5 from methylated and unmethylated alleles. Background intensity, calculated from negative controls, was subtracted from each data point. Data points were then analysed using beta values and M values. Beta values represent the ratio of methylated probe intensity to the total intensity, derived from the sum of methylated and unmethylated probe intensities. M values are calculated as the log2 ratio of methylated to unmethylated probe intensities. Raw methylation data were preprocessed with the minfi package (260) and data quality was evaluated using the minfi and RnBeads packages (261,262). After Snoob normalisation, M values were used to obtain adjusted p-values (Benjamini-Hochberg FDR) between sample groups via an eBayes-moderated paired t-test using the limma R package (263). Differentially methylated CpGs were identified based on an FDR < 0.05 and a beta value change greater than 5%. Hierarchical clustering

with Pearson correlation distances and average linkage criteria, along with DMP heatmaps, were generated using functions from the gplots and ComplexHeatmap R packages.

4.10 DNA Methylation Data Analysis

In order to assess enrichment of transcription factor motifs in our DNA methylation dataset we used the HOMER software (264). Specifically, we used the findMotifsGenome.pl algorithm (with settings -size 250 -cpg) to individuate significant enrichment against a background sequence adjusted to have similar CpG and GC contents. Genomic regions were annotated with the annotatePeaks.pl algorithm. To assess the position relative to a CpG island, we used 'hg19_cpgs' annotation in the annotatr R package. GREAT software (265)vv was used to obtain gene ontologies by using the single nearest gene option to define associations between genomic regions and coding genes. Chromatin functional state enrichment of DMPs was analysed using as background public available CD14 primary cells data obtained from the NIH Roadmap Epigenomics Project (http://www.roadmapepigenomics.org) generated with the ChromHMM software (266) by using a 15-state model — primary HMM — constructed with data from 5 histone modification marks and checking for enrichment and significance by Fisher's exact tests.

4.11 Bulk RNAseq analysis

Starting from total RNA obtained from either monocytes, mDCs or different types of tolDCs from HD and MS patients, RNA-seq libraries were generated and sequenced by Novogene (Cambridge). Samples were sequenced in 150-bp paired-end using an Illumina NovaSeq 6000 machine and at least 40 million

reads were obtained for each sample. Fastq files were aligned to the hg38 transcriptome using HISAT2 (267) and reads mapped in proper pairs and primary alignments were selected with SAMtools (268). Then, reads were assigned to genes with featureCounts (269) and differentially expressed genes (DEGs) were calculated with DESeq2 (270). The Normal shrinkage algorithm was used and genes with an FDR < 0.05 and a Log2FC > ± 0.5 were considered DEGs.

4.12 RNAseq Data Analysis

Inference of transcription factors activity from gene expression values were assessed using DoRothEA (271).

4.13 Cytokine Quantification of culture supernatants

The production of IL-6, IL-12p70, IL-1β was quantified simultaneously at day 6 of differentiation in the culture supernatants of tolDC, tolDC FICZ and tolDC DMF by using LEGENDplexTM Human Essential Immune Response Panel according to manufacturer's instructions. Samples were acquired on an LSR Fortessa flow cytometer (BD Biosciences) and analysed using FACSDiva software. Quantification of IFNγ, TNF-α and IL-1β in allogeneic MLRs supernatants was performed with the same technique at day 6 of co-culture.

4.14 Metabolic Analysis of Culture Supernatants

Glucose consumption, secretion of lactate and pH quantification were performed on supernatants collected on day 6 of differentiation to tolDCs,

tolDCs + FICZ or tolDCs + CH223191. Glucose and lactate concentrations were determined in an AU5800 platform (Bekman Coulter; Clare, Ireland) using a standard hexokinase method and a lactate oxidase reaction, respectively. For pH quantification, a direct potentiometry method was used in a Gem Premier 4000 analyzer (Werfen, MA, USA).

4.15 Mice

Female and male C57BL/6J mice, aged 8–10 weeks, were obtained from Envigo Rms Spain SL (Sant Feliu de Codines, Barcelona, Spain) and housed at the Comparative Medicine and Bioimage Centre of Catalonia (CMCiB). They were kept under standard light and climate-controlled conditions, with free access to a standard chow diet and water.

4.16 Bone Marrow-Derived Dendritic Cell Differentiation

Bone marrow-derived dendritic cells (BMDCs) were generated as previously described by isolating progenitor bone marrow cells from the femurs and tibiae of C57BL/6 donor mice and culturing them in RPMI medium supplemented with 2% L-Glutamine (Sigma-Aldrich), 10% FBS, 1% Sodium pyruvate (Sigma-Aldrich), and 1% penicillin-streptomycin (Thermo-Fisher Scientific) in the presence of 1000 IU/mL of murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech). For the development of VitD3-tolDCs, 500 IU/mL Calcitriol (Kern Pharma) was added for 8 days. On day 7, 0.1 mg/mL of lipopolysaccharide (LPS) (Sigma-Aldrich) was added to the culture medium of mDCs and VitD3-tolDCs for 22-24 hours. Then, 10 µM myelin oligodendrocyte glycoprotein 35-55 (MOG35-55) peptide was added for 18 hours to pulse the DCs. Finally, the VitD3-tolDC-MOG cells were cryopreserved in batches of 10^7

cells and stored in liquid nitrogen until needed. The VitD3-tolDC-MOG were characterized by assessing their phenotype and functionality using a mixed allogeneic lymphocyte reaction suppression assay.

4.17 Induction of EAE and Clinical Follow-Up

EAE was induced by subcutaneously immunizing the mice with 100 µg of MOG35–55 (YRSPFSRVVHLYRNGK) (Immunostep, Salamanca, Spain), emulsified in an equal volume (1:1) of Freund's complete adjuvant containing 4 mg/mL Mycobacterium tuberculosis (strain H37RA, Difco, Detroit, MI, USA). Additionally, mice received an intravenous injection of 250 ng pertussis toxin (Sigma Chemical, St. Louis, MO, USA) on day 0 and day 2 post-immunization (dpi). Animals were weighed and monitored daily for welfare and clinical signs. Clinical evaluation was based on the following criteria: 0, asymptomatic; 0.5, loss of distal half of tail tone; 1, loss of entire tail tone; 1.5, hind limb weakness; 2, hind limb paralysis; 2.5, hind limb paraplegia; 3, forelimb weakness; 4, quadriparesis; 4.5, severe quadriparesis; 5, quadriplegia; and 6, death. Endpoint criteria were established to minimize suffering and ensure animal welfare.

4.18 Treatment of EAE Mice with tolDCs or DMF

For the DMF treatment, mice were treated daily with vehicle 1 (methylcellulose) or DMF (100 mg/kg body weight, Sigma-Aldrich) suspended in 0,8% methylcellulose (Sigma-Adrich). DMF or vehicle 1 was administered through oral gavage from dpi 3 until the end of the experiment. Regarding the VitD3-tolDC treatment, vehicle 2 (PBS) or 1x10^6 VitD3-tolDC-MOG was administered intravenously on dpi 13, 17, 21 and 24. In order to compare the combined therapy with both mono-therapies, the different groups of treatment

were:vehicle 1 and vehicle 2, DMF and vehicle 2, VitD3-tolDC-MOG and vehicle 1, and DMF and VitD3-tolDC-MOG.

4.19 Infiltrating Lymphocyte analysis

Spinal cords were collected on dpi 24 by flushing them from the spinal column with PBS. After mechanical disaggregation and enzymatic digestion with 1mg/ml DNase I (11284932001, Roche) and 1 mg/ml collagenase A (C2674, Sigma-Aldrich) for 30 min at 37°C, myelin debris was removed using a Percoll gradient centrifugation. The cells were resuspended with 30% Percoll, added onto a 70% Percoll solution, and centrifuged at 500 x g for 20 min at room temperature. Cells were collected at the interface. Next, for the intracellular staining of infiltrating lymphocytes, the cells were stimulated with 25 ng/mL PMA (P8139, SigmaAldrich) and 1 µg/mL ionomycin (I3909, Sigma-Aldrich) for 4h. During the last 2h of incubation the 100 ug/ml brefeldin A (B5936, Sigma-Aldrich) was added. Cells were then blocked with anti-mouse CD16/32 (14-0161-85, Invitrogen) and dead cells were labelled with Fixable Viability Stain 575V (565694, BD Biosciences). We then performed surface staining with antibodies against CD3 (100306, Biolegend) and CD4 (46–0042, eBiosciences565650, BD Pharmigen). Cells were fixated for 15 min with 2% PFA and kept in FacsFlow overnight. Intracellular staining of cytokines was performed to detect IL-17A (563354, BD Horizon), IFNγ (554413, BD Pharmingen), IL-4 (560699, BD Pharmingen), and IL-10 (554467, BD Pharmingen). First, cells were permeabilized with Permeabilization Buffer (00-8333-56, Invitrogen) and incubated for 30 min at 4°C with the antibody mix. Samples were acquired in a BD LSRFortessa flow cytometer and data was analysed with the OMIQ software.

4.20 Analysis of regulatory T cells in mouse splenocytes

Suspension of murine splenocytes was obtained by grinding the spleens through a 70µm nylon cell strainer at dpi 24. Cells were labeled with Fixable Viability Stain 575V. To analyse Treg cell population in the spleen, antibodies against CD3 (100306, Biolegend), CD4 (560468, BD Horizon), CD25 (558642, BD Pharmigen), and Foxp3 (560401, BD Pharmigen) were used. After staining the surface markers, the manufacturer's instructions were followed for the intracellular labelling of Foxp3 using the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (00-5523-00, Invitrogen). Samples were acquired in a BD LSRFortessa flow cytometer and data was analysed with the OMIQ software.

4.21 Antigen-Specific T Cell Reactivity

To study antigen-specific reactivity, splenocytes from all treatment groups were cultured in a 96-well plate at 1,5 × 10^5 cells/well in 200 µL of IMDM (supplemented with 10% FBS, 2% L-Glutamine, 1% penicillin/streptomycin and 1% sodium pyruvate) containing either 5 µM MOG35-55, 5 µM phytohemagglutinin (PHA) (Sigma-Aldrich) (positive control) or culture medium (negative control). After 72 h of culture, 1 µCi/well of [3H]-thymidine (PerkinElmer) was added for the last 18 h of culture. The stimulation index (SI) for each stimulus was calculated as the mean counts per minute (cpm) of antigen-stimulated cultures divided by the mean cpm of the non-stimulated cultures.

4.22 Sex as a biological variable

The MS and HD cohorts for RNAseq and DNA methylation analysis were designed to be matched by sex and age. When comparing HD and MS patients in MLRs, samples were matched by sex and age. Our study included both male and female animals, with similar findings observed in both sexes.

4.23 Statistical Analysis

All statistical analyses were performed using Prism 9.0 software (GraphPad, La Jolla, CA, USA) or R software v 4.3.1, with either parametric or non-parametric tests depending on the normality of the dataset. Exact statistical tests are reported in figure captions. Results were shown in plots as mean ± SD, unless noted differently and with exact p-values.

4.24 Study approval

This study was approved by the Germans Trias i Pujol Hospital ethics committee, and all patients and healthy donors signed an informed consent. For experiments involving mice, the experimental procedures were approved by the Ethics Committee of Animal experimentation of Comparative Medicine and Bioimage Centre of Catalonia (CMCiB) and the Generalitat of Catalonia (Spain). In experiments involving buffy coats, anonymous blood samples were obtained through the Banc de Sang i Teixits (Barcelona, Spain), following the institutional Standard Operating Procedures for blood donation following the principles of the World Medical Association (WMA) Declaration of Helsinki, which included a signed informed consent.

4.25 Data and code availability

DNA methylation and RNA-seq data associated with this publication have been deposited in the NCBI Gene Expression Omnibus and can be accessed via GEO Series accession numbers GSE267660 and GSE267576, respectively.

5. Results

5.1 Monocytes from MS patients are characterised by an activated phenotype

To investigate the potential effect of systemic inflammation on circulating monocytes of MS patients, we first studied the peripheral blood CD14+ fraction isolated from naive, active relapsing-remitting MS patients and healthy donors (HD) using a multi-omic approach involving spectral flow cytometry, DNA methylation arrays and bulk RNAseq (Fig. 1a).

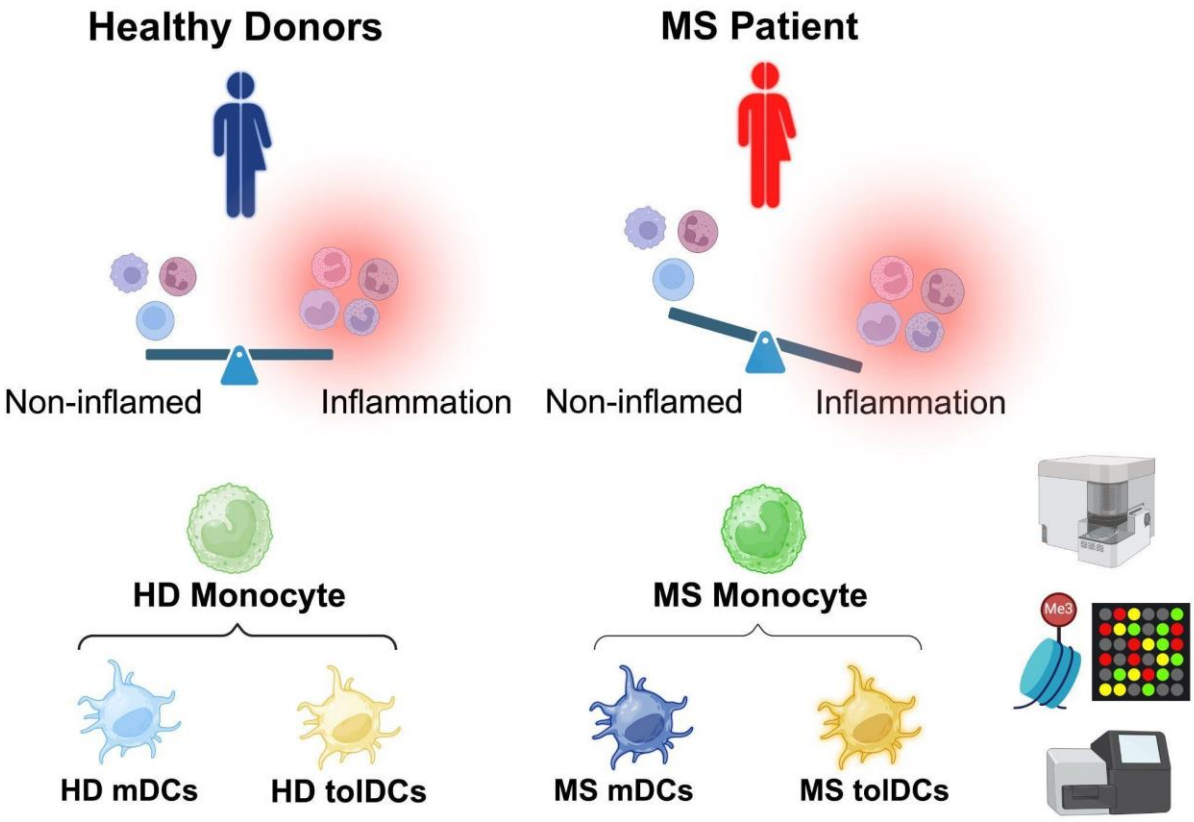
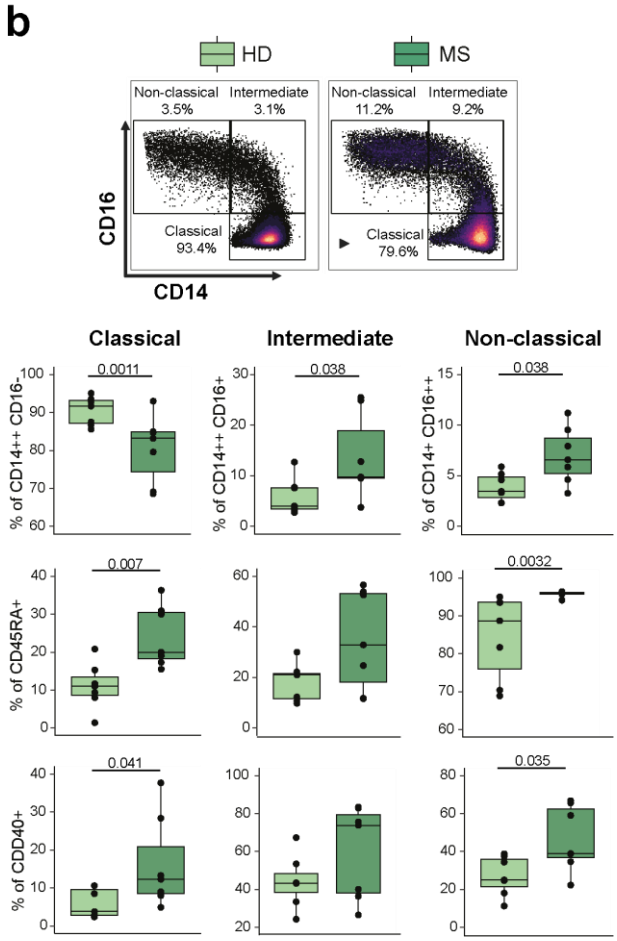


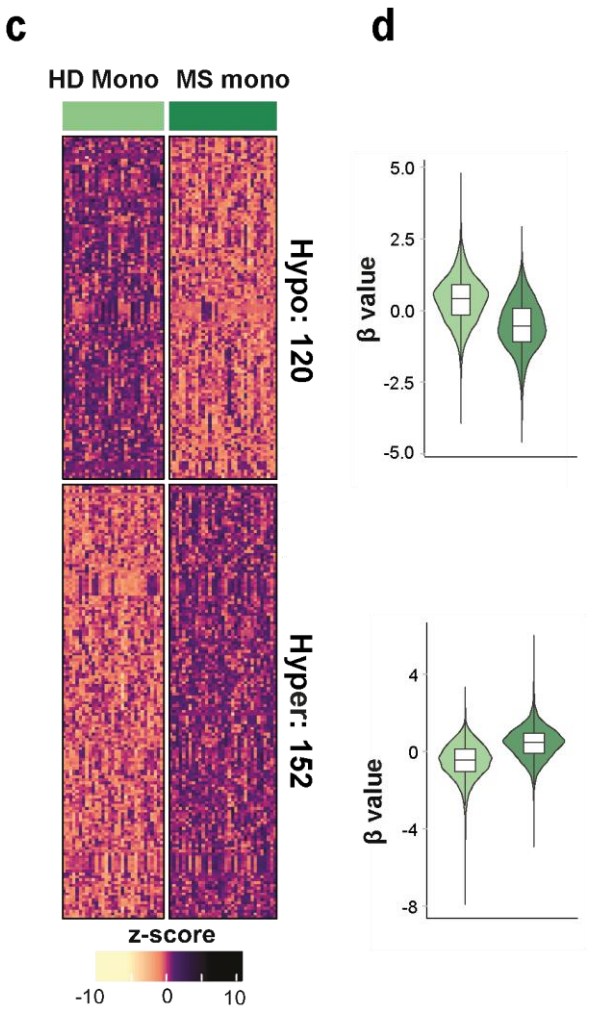
Fig.1a: Schematic overview of the experimental model from MS or HD-derived peripheral blood Monocytes, mDCs and tolDCs.

Spectral flow cytometry analysis showed a prominent increase of non-classical (CD14+CD16++) and intermediate monocytes (CD14++CD16+) at the expense of classical monocytes (CD14++CD16-) in MS patients (Fig. 1b). This shift in monocyte subsets was also accompanied by higher expression in classical and non-classical subsets of the surface markers CD45RA and CD40 (Fig. 1b), both of which are increased in monocytes in other inflammatory conditions and are considered activation markers (272–275).



(1b) Flow cytometry representative figures and boxplots reporting different percentages of Classical (CD14++ CD16-), Intermediate (CD14+ CD16+) and non-classical (CD14+ CD16++) monocytes among MS patients and HD, with respect to total monocytes as parent gate (first row), or reporting the percentage of CD45RA (second row) or CD40 (third row) with respect to Classical, Intermediate or Non-Classical monocytes. *P*-values from Mann-Whitney test are shown in figures in case of statistical significance.

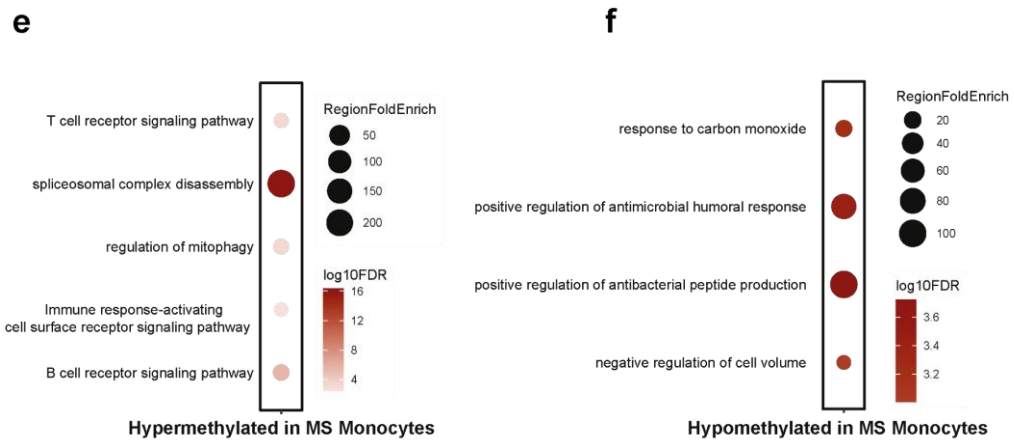
Given the critical role of DNA methylation in myeloid function and the known changes in monocytes in relation to disease activity in other inflammatory diseases(253–255), we profiled DNA methylation of CD14+ monocytes obtained from MS patients (MS monocytes) and HD (HD monocytes) using Illumina Infinium MethylEPIC arrays. Using this method, we tested the methylation status of 831,421 CpGs in the human genome. The comparison between MS and HD monocytes showed the existence of differentially methylated positions (DMPs) comprising 120 hypomethylated and 152 hypermethylated (false discovery rate < 0.05 and absolute differential beta (Δβ) > 0.05) (Fig. 1c, 1d), supporting that the DNA methylation profiles of monocytes are also affected during the active phase of MS.

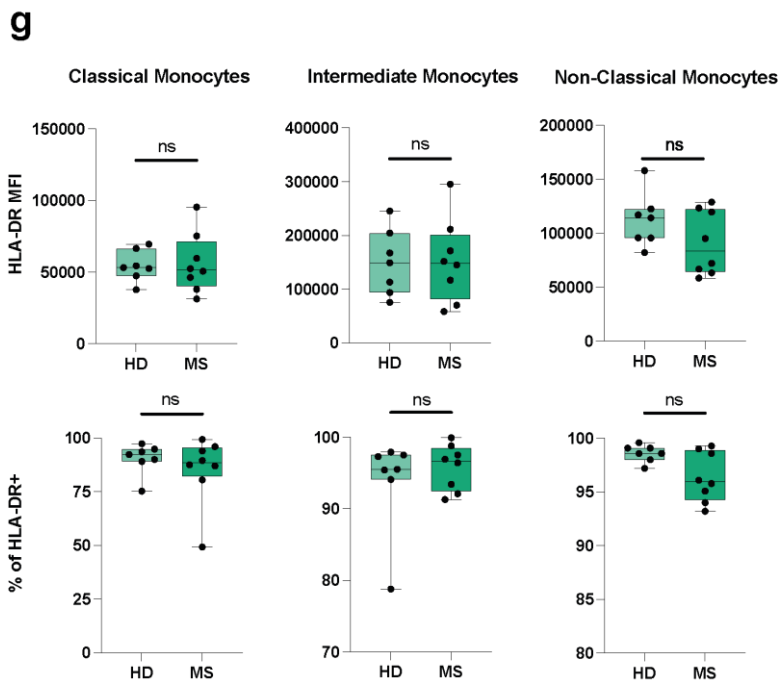


(1c) DNA methylation heatmap of 18 vs 18 samples of HD and MS Mono. The heatmap includes all CpG-containing probes displaying significant methylation changes (from now on

called DMPs) (q value < 0.05 and β > 0.05) in the HD Mono - MS Mono contrast. **(1d)** Violin plots showing the general distribution of DNA methylation across clusters of hyper or hypomethylation in the HD Mono - MS Mono contrast.

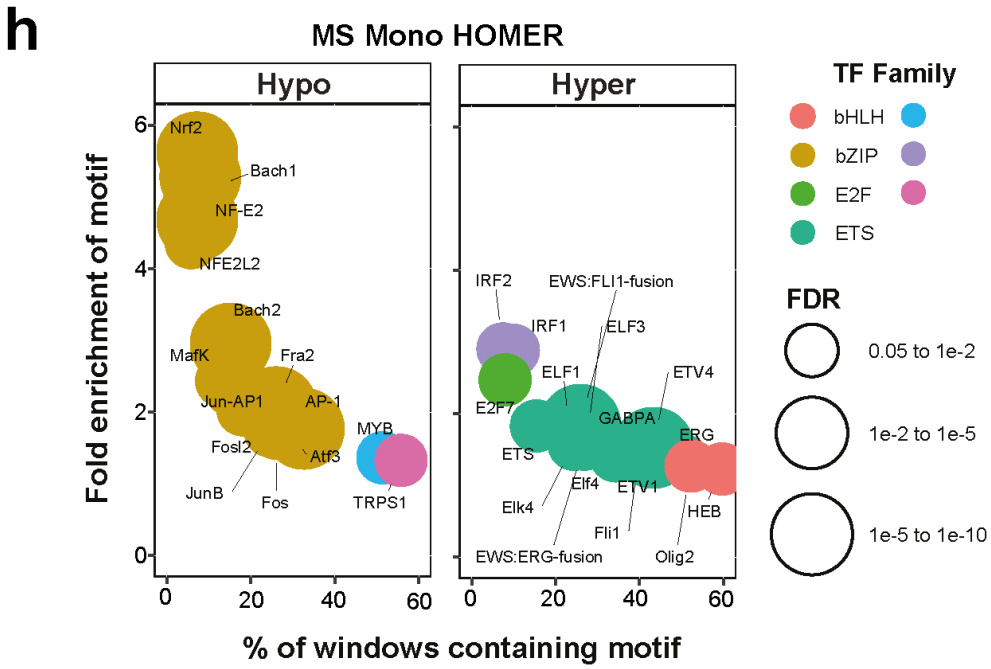
Functional Gene Ontology (GO) Analysis (Fig. 1e) of the hypermethylated DMPs cluster showed significant enrichment of categories linked to antigen presentation and regulation of the adaptive immune response, while the hypomethylated DMPs cluster was mainly represented by pathways linked to positive regulation of humoral immunity (Fig. 1f). No changes in HLA-DR protein expression were observed among HD and MS monocytes (Fig. 1g).





(1e) and **(1f)** Gene ontology (GO) terms associated with CpGs from hypermethylated (a) and hypomethylated (b) clusters as analysed by GREAT software. Selected categories are shown. Bars represent log-transformed binomial q values of the GO term enrichment for the HD Mono - MS Mono contrast. **(1g)** Flow cytometry boxplots reporting Median Fluorescent intensity (MFI) of HLA-DR expression in Classical (CD14++ CD16-), Intermediate (CD14+ CD16+) and Non-classical (CD14+ CD16++) monocytes among MS patients and HD, with respect to total monocytes as parent gate (top row), or reporting the % of HLA-DR-positive (second row) monocytes with respect to Classical, Intermediate or Non-Classical subpopulation. *P*-values from Mann-Whitney test are shown.

Next, we checked for enrichment of transcription factor (TF) binding motifs spanning 250 bp in each direction from differentially hyper or hypomethylated DMPs (Fig. 1h) using HOMER (264).

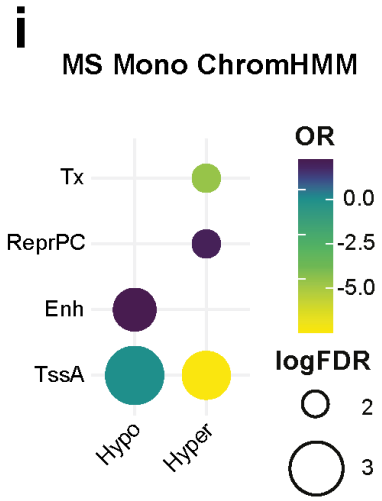


(1h) Bubble scatterplot HOMER analysis of significantly enriched transcription factors motifs in the hypermethylated and hypomethylated clusters regions in HD-MS Mono contrast. The x-axis shows the percentage of windows containing the motif, while the y-axis the fold enrichment of the motif over background. Bubbles colours indicate different TF families, while their size is proportional to the false discovery rate (FDR).

The hypermethylated cluster was enriched in the binding motifs of TFs-linked to type-I Interferon (IFN) response and the inflammasome (IRF1, IRF2), immune cell differentiation (ERG) and transcriptional regulation (ELF1, ETV2, ETV4).

ETV2 and ETV4 belong to the same family of TFs as ETV3 and ETV6 and are crucial in determining IFN responses and fate commitment to mo-DCs vs mo-macrophage (276). Moreover, ETV6 is a therapeutic target in experimental autoimmune encephalitis (EAE) mice, and its deletion in myelin-presenting pathogenic DCs contributed to ameliorating the clinical score of mice (269). The hypomethylated DMP cluster was significantly enriched in the binding motifs of TFs regulating monocyte-to-macrophage differentiation (MYB), NF-kB (JunB, Fosl, AP-1), the magnitude of IFN-beta production in innate immune cells (ATF3), and of NRF2 (NRF2, NFE2LF), a basic leucine zipper transcription factor induced by metabolic or oxidative stress triggered by inflammation (277), which positively regulates the expression of anti-inflammatory molecules.

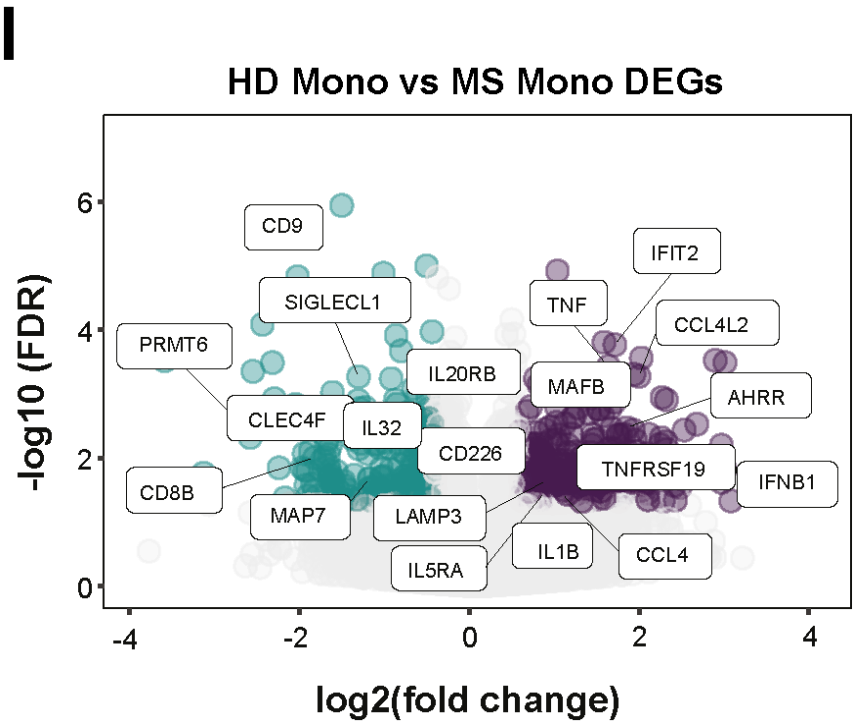
We then profiled the association of hyper and hypomethylated DMPs at 18 distinct chromatin states using ChromHMM (266).



(1i) Chromatin functional state enrichment analysis of the differentially hyper and hypomethylated probes in the HD Mono vs MS Mono contrast based on CD14+ primary cells ChromHMM public data from Roadmap Epigenomics Project. Odds Ratio is reported on a colour scale, while the size of the bubble is proportional to Log of the *False Discovery Rate (*FDR). Significant enriched categories are shown (FDR < 0.05, odds ratio > 2), including Tx (strong transcription), ReprPC (repressed PolyComb), Enh (enhancers), TssA (Active Transcription Starting Site).

We observed a significant enrichment of regions of active transcription start sites and enhancers (Fig. 1i) with respect to background in the hypomethylated DMPs, and of active transcription start sites and repressors in the hypermethylated DMPs, suggesting a direct connection between methylation status and the transcription of genes associated to differentially hypomethylated CpGs. Overall, MS monocytes presented an altered DNA methylation profile in comparison to HD monocytes, skewed toward a proinflammatory and activated profile.

The analysis of bulk RNAseq data (Fig. 1l, left, downregulated genes; right, upregulated genes, in the comparison between MS monocytes and HD monocytes) also supported the acquisition of a transcriptomic signature in MS monocytes compatible with a pro-inflammatory phenotype.

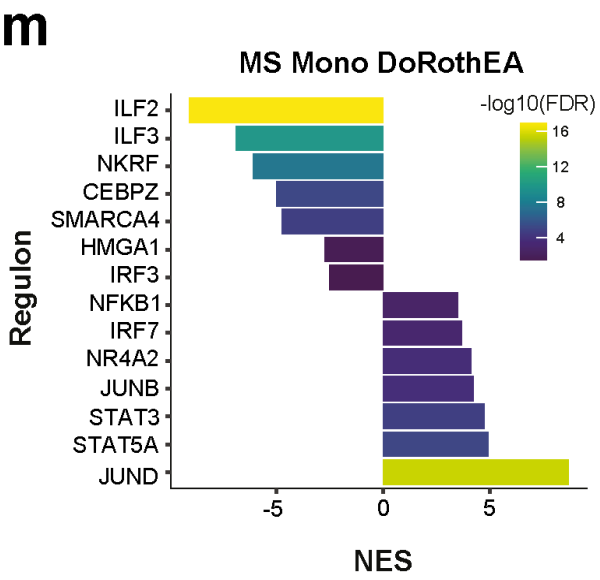


(1l) Volcano plots of gene expression showing Mono HD - Mono MS contrast, with binary logarithm of the fold change on the x-axis and the negative decimal logarithm of the FDR on the y-axis. Differentially downregulated and upregulated genes are shown if False Discovery Rate < 0.05 and Log2 Fold Change < –0.5 and False Discovery Rate < 0.05, Log2 Fold

Change > 0.5. Turquoise bubbles represent downregulated genes, while violet bubbles upregulated genes.

The comparison of the RNAseq profiles between MS and HD showed 333 overexpressed and 248 downregulated genes (Log2 Fold change > ± 0.5, False Discovery Rate < 0.05). These include the upregulation of key inflammation-related genes such as *TNF*, *IFNB1, CCL4*, and *AHRR*, encoding the repressor of the AhR, a TF that is key in the acquisition of the tolDC phenotype. Moreover, we observed downregulation of the methyltransferase PRMT and MAP7, a molecule previously described by our group as a biomarker of VitD3-tolDCs, which can be used to distinguish these cells from iDCs) and mDCs (241).

Lastly, we pinpointed transcription factors that may contribute to the transcriptomic alterations observed in monocytes from individuals with MS, employing Discriminant Regulon Expression Analysis (DoRothEA) (271) on our RNAseq dataset.

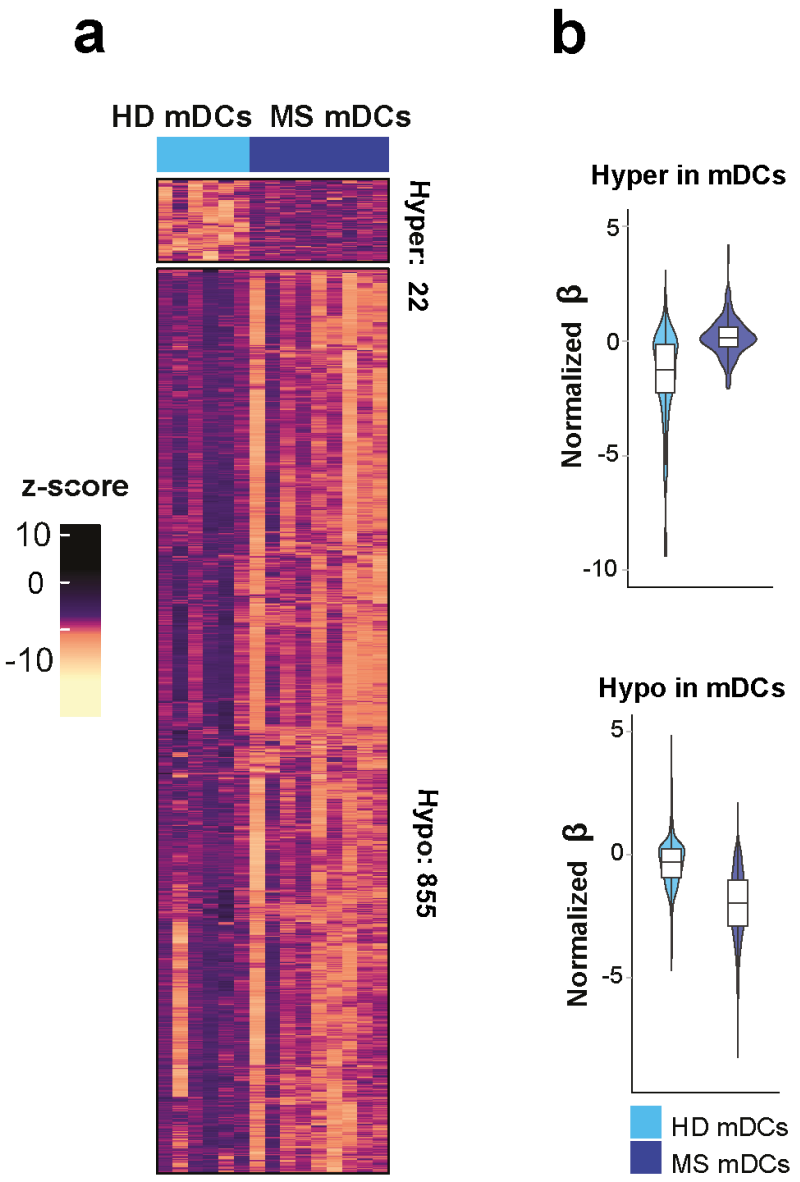


(1m) Bar plot depicting the transcription factor activity predicted from mRNA expression of target genes with DoRothEA v2.0 in the HD vs MS Mono contrast in terms of normalised enrichment score (NES). Regulons with a a high confidence score of A–B were analysed, and cases with $p < 0.05$ NES of ± 2 were considered significantly enriched.

MS Mono showed positive enrichment of several pivotal inflammatory factors (Fig. 1m), such as NFkB, STAT3, STAT5A and IRF7. Interestingly, MS monocytes also showed a significant depletion of NFKB repressing factor NKRF, and of ILF2 and ILF3, which are involved in suppressing the acquisition of a mature phenotype in the monocyte-to-DC axis (278) and DC-mediated immune responses. In conclusion, multi-layer analysis of protein expression, transcriptome and epigenome determined that MS monocytes display a pro-inflammatory phenotype in comparison to HD monocytes, defined by increased activation of canonical inflammation pathways.

5.2 The pro-inflammatory signature is maintained in monocyte-derived mDCs and tolDCs from MS patients

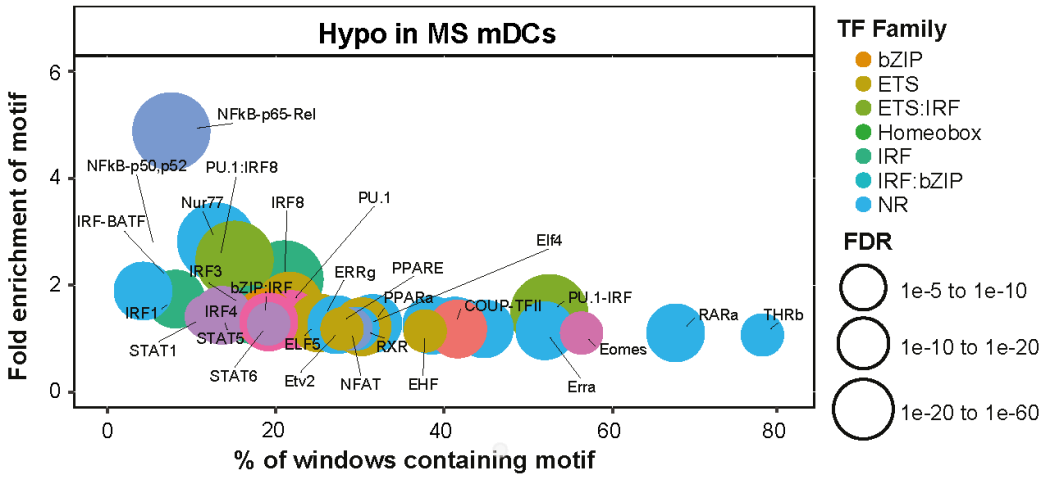
To test our hypothesis that MS-intrinsic inflammatory footprint on CD14+ monocytes is retained after differentiation into monocyte-derived DCs, we conducted DNA methylation profiling and bulk RNAseq of HD- and MS-derived mDCs and tolDCs. mDCs and tolDCs from MS patients and HD monocytes were differentiated in vitro for 6 days using GM-CSF, IL-4, TNF-α, PGE2, and IL-1β, either in the absence or presence of Vitamin D3 as a tolerizing agent. The DNA methylation profiles of MS mDCs displayed differences in comparison to HD mDCs (Fig. 2a and Fig. 2b) that mainly consisted of a big cluster of hypomethylation (Hypomethylated DMPs = 916; Hypermethylated DMPs = 57, FDR < 0.05 and $\Delta\beta > 0.05$).



(2a) DNA methylation heatmap of 6 vs 8 samples of HD and MS mDCs. The heatmaps include all CpG-containing probes displaying significant methylation changes (DMPs) (q value < 0.05, beta > 0.05) in the HD mDCs-MS mDCs contrast. **(2b)** Violin plots showing the general distribution of DNA methylation across hyper or hypomethylated clusters in HD mDCs and MS mDCs.

Like the results obtained with MS monocytes, HOMER analysis of the MS mDCs hypomethylated DMPs showed an enrichment of binding motifs of key inflammatory TFs such as NFkB, p65, STAT1, STAT5, STAT6, IRF1, IRF3, and IRF4, suggesting a more immunogenic phenotype of MS-derived mDCs (Fig. 2c).

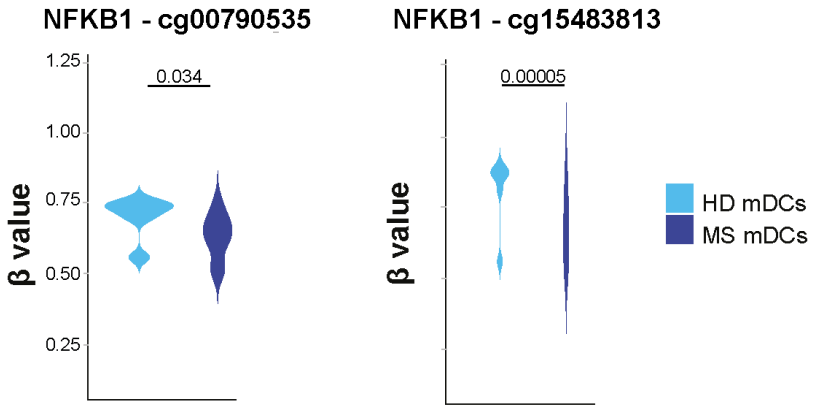
c



(2c) Bubble scatterplot HOMER analysis of significantly enriched transcription factors motifs in the hypermethylated and hypomethylated clusters regions in HD - MS mDC contrast. The x-axis shows the percentage of windows containing the motif, while the y-axis the fold enrichment of the motif over background. Bubbles colours indicate different TF families, while their size is proportional to the false discovery rate (FDR).

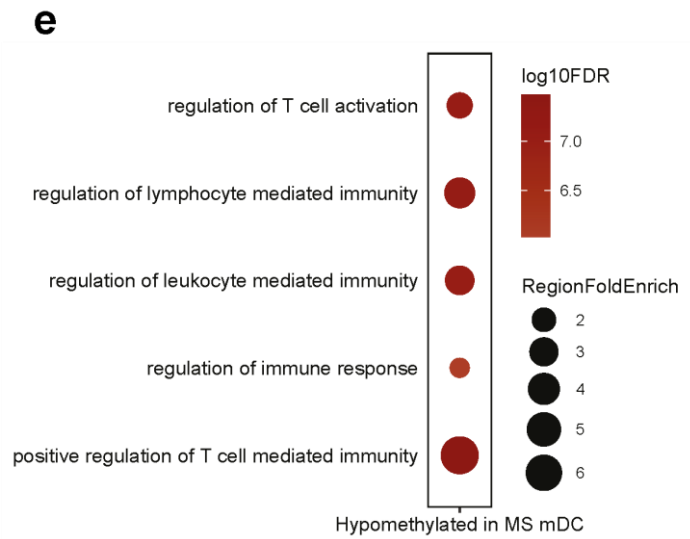
We detected hypomethylation of 2 CpGs mapping at the NFKB1 gene (Fig. 2d). On the other hand, there was no significant enrichment of TF binding motifs in the hypermethylated DMP cluster.

d



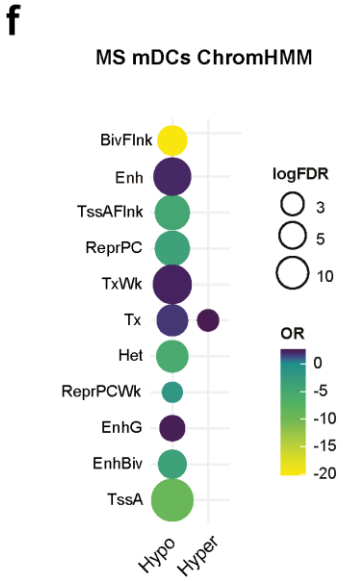
(2d) Violin plots showing DNA methylation levels (*β*-values) of NFKB1 individual CpGs in HD mDCs - MS mDCs comparisons. P-values correspond to false discovery rate (significant if FDR < 0.05).

Functional GO analysis (Fig. 2e) of the hypomethylated cluster showed enrichment of categories linked to activation of the adaptive immune response.



(2e) Gene ontology (GO) terms associated with CpGs from hypomethylated as analysed by GREAT software. Selected categories are shown. Bars represent log-transformed binomial q values of the GO term enrichment for the HD mDCs - MS mDCs contrast.

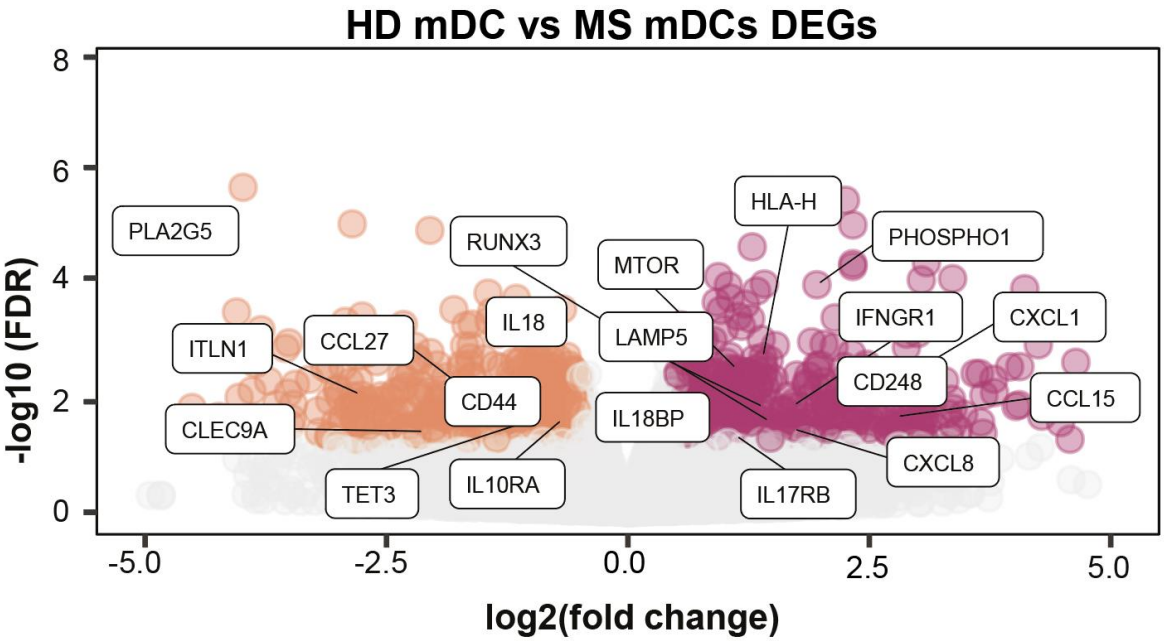
In addition, ChromHMM pointed out enrichment in active transcription start sites, enhancers and repressors for the hypomethylated DMPs (Fig. 2f).



(2f) Chromatin functional state enrichment analysis of the differentially hypomethylated probes in HD-MS mDC contrast, based on CD14+ primary cells ChromHMM public data from Roadmap Epigenomics Project. Odds Ratio is reported on a colour scale, while the size of the bubble is proportional to LogFDR. Significant enriched categories are shown (FDR < 0.05,

odds ratio > 2), including TssA, active TSS; TssAFlnk, flanking active TSS; Tx, strong transcription; TxWk, weak transcription; EnhG, genic enhancers; Enh, enhancers; Het, heterochromatin; TssBiv, bivalent/poised TSS; BivFlnk, flanking bivalent TSS/Enh; EnhBiv, bivalent enhancer; ReprPC, repressed PolyComb; ReprPCWk, weak repressed PolyComb.

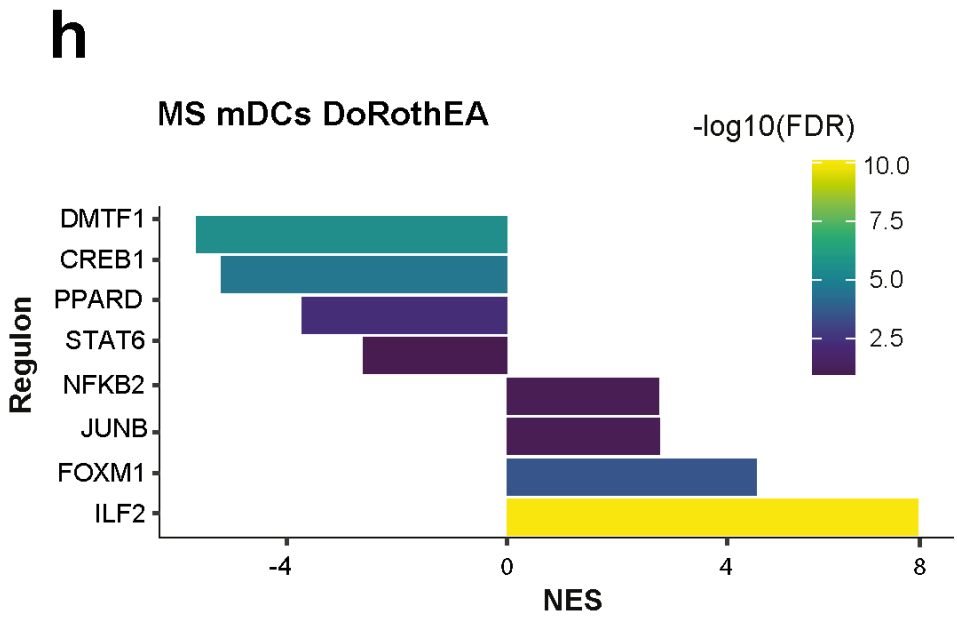
In parallel, RNAseq data (Fig. 2g, left, downregulated genes; right, upregulated genes in HD mDCs vs MS mDCs) also revealed an increase in inflammatory pathways: there was upregulation of genes encoding CXCL1, IL-8 (CXCL8) and IL-27, three cytokines produced by activated DCs which regulate inflammatory responses and are regulated by NFkB signalling (279–283) and mTOR, which plays a central role in regulating DC differentiation, immune responses and autophagy (284).



(2g) Volcano plots of gene expression showing HD-MS mDCs contrast, with the binary logarithm of the fold change on the x-axis and the negative decimal logarithm of the FDR on the y-axis. Differentially downregulated and upregulated genes are shown if False Discovery Rate < 0.05 and Log2 Fold Change < –0.5 and False Discovery Rate < 0.05, Log2 Fold Change > 0.5. Orange bubbles represent downregulated genes, while magenta bubbles upregulated genes.

On the other hand, MS mDCs expressed less of CD300LB, a molecule regulating DC efferocytosis (285), IL-18, a cytokine inducing Th1 responses ,

and CLEC9A, a C-type lectin receptor involved in the recognition of necrotic cells and antigen uptake (286). Finally, MS mDCs showed a positive enrichment of NFkB and ILF2, a factor linked to the regulation of IL-2 production, and a negative enrichment of PPARD, the receptor of PPARγ, a soluble factor involved in inducing Th2 responses (Fig. 2h).

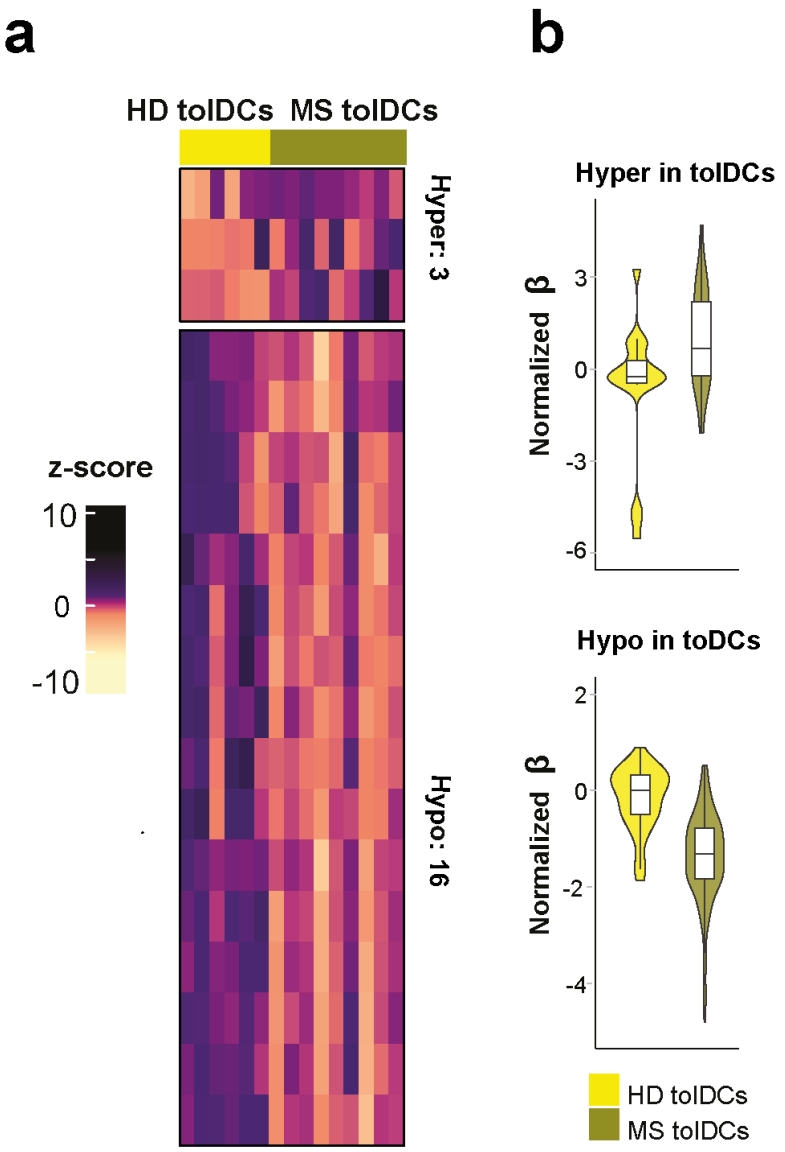


(2h) Bar plot depicting the transcription factor (TF) activity predicted from mRNA expression of target genes with DoRothEA v2.0 in the HD-MS mDCs contrast in terms of normalised enrichment score (NES). Regulons with a high confidence score of A–B were analysed, and cases with $p < 0.05$ NES of ± 2 were considered significantly enriched.

Overall, MS mDCs appeared to have a more immunogenic profile in comparison to HD, mainly characterised by the activation of the NFkB pathway.

5.3 Vitamin D tolerization do not revert MS DCs inflammatory fingerprint

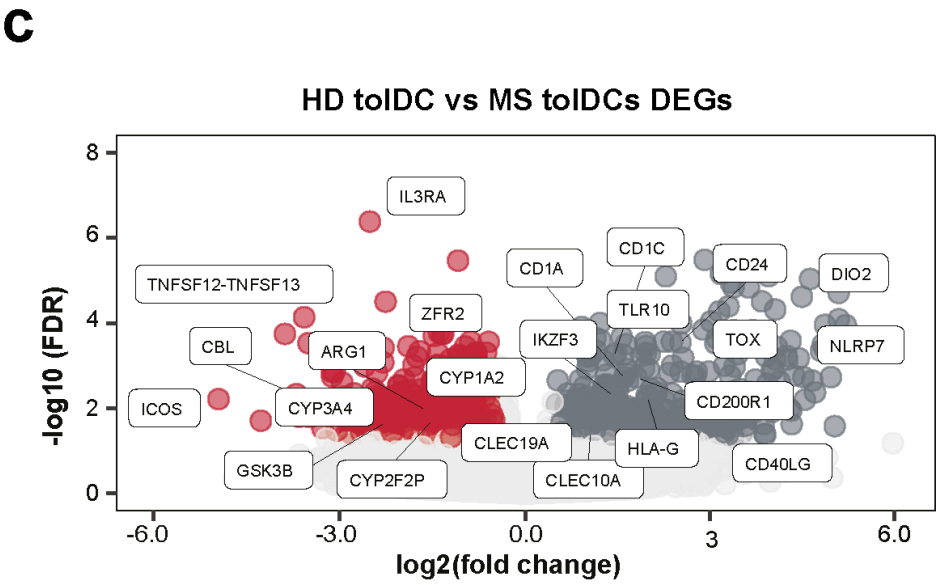
In contrast with MS mDCs, MS tolDCs did not show wide DNA methylation changes in comparison to HD tolDCs (Fig.3a and 3b), with very few DMPs present in this comparison.



(3a) DNA methylation heatmap of 6 vs 8 samples of HD and MS tolDCs. The heatmaps include all CpG-containing probes displaying significant methylation changes (DMPs) (q value < 0.05,

beta > 0.05) in the HD tolDCs-MS tolDCs contrast. **(3b)** Violin plots showing the general distribution of DNA methylation across hyper or hypomethylated clusters in HD tolDCs and MS tolDCs.

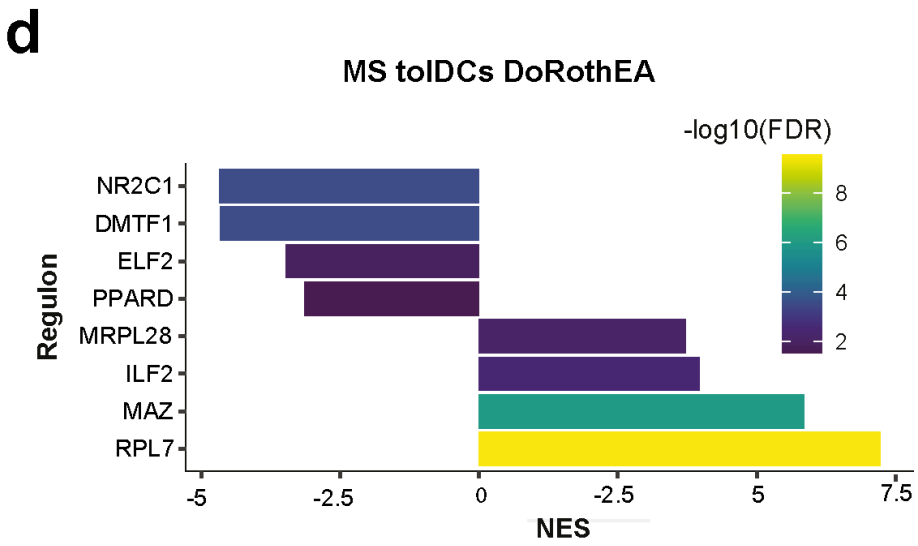
On the other hand, MS tolDCs still showed conspicuous changes at the transcriptomic level (Fig. 3c), with an increased expression of the activation markers CD1c, CD1a, CD24 and CD40LG and reduced expression of the immune checkpoint ICOS and CYP1A2 encoding genes. The latter is used together with CYP1A1 as a surrogate marker to infer *AHR* activity, which is also involved in monocyte-to-DC differentiation, in addition to the acquisition of tolerogenic features (197,287,288).



(3c) Volcano plots of gene expression showing HD-MS tolDCs contrast, with the binary logarithm of the fold change on the x-axis and the negative decimal logarithm of the False Discovery Rate (FDR) on the y-axis. Differentially downregulated and upregulated genes are shown if FDR< 0.05 and Log2 Fold Change < –0.5 and FDR < 0.05, Log2 Fold Change > 0.5. Red bubbles represent downregulated genes, while grey bubbles upregulated genes.

Additionally, MS tolDCs expressed less ARG1, involved in conferring immunosuppressive properties to tolDCs (289). Regulon analysis using DoRothEA showed a negative enrichment of PPARD and positive enrichment of ILF2, as observed in MS mDCs (Fig. 3d). Taken together, despite the few

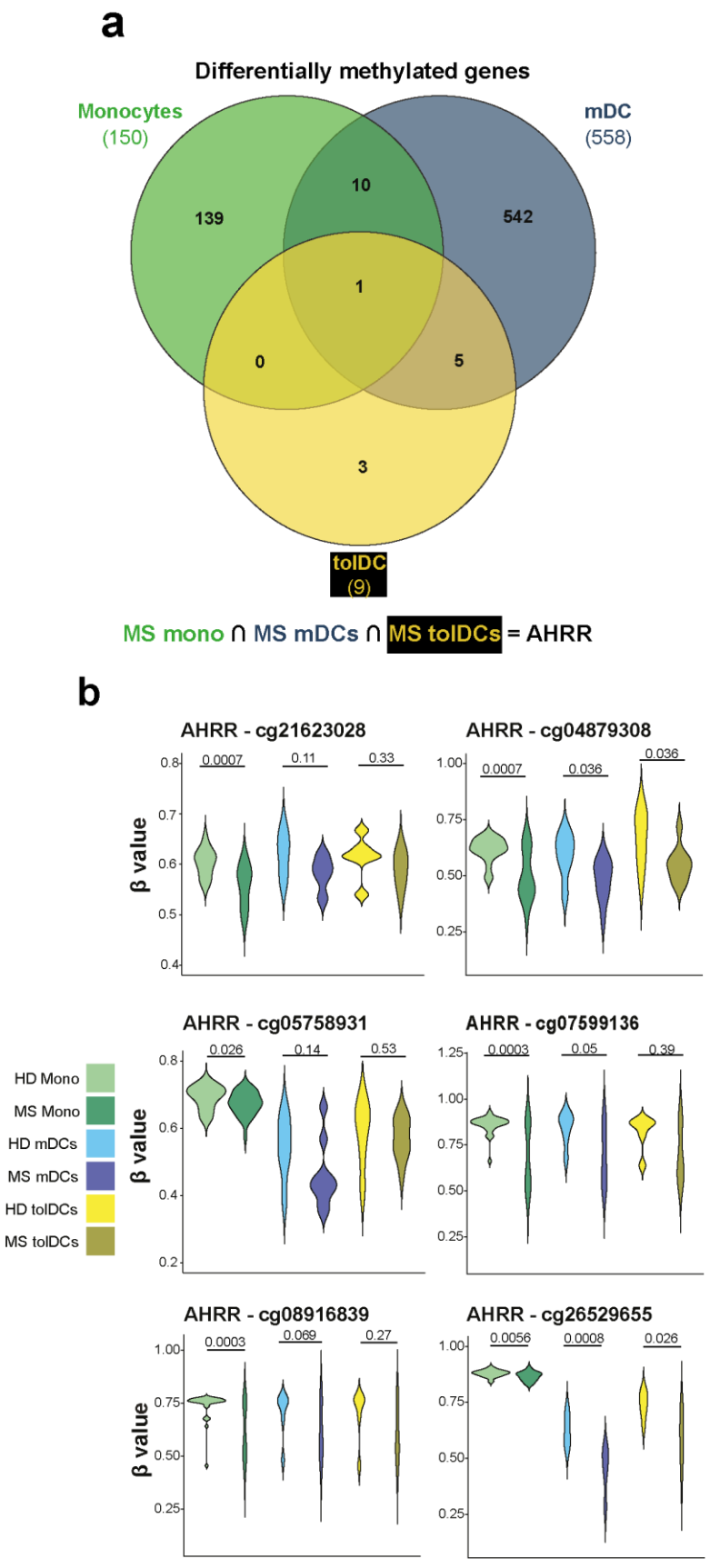
differences at the DNA methylation level, MS tolDCs appear to have a more mature and activated profile at the transcriptomic level, with several tolerogenic pathways that are downregulated in comparison to HD tolDCs.



(3d) Bar plot depicting the transcription factor activity predicted from mRNA expression of target genes with DoRothEA v2.0 in the HD-MS tolDCs contrast in terms of Normalised Enrichment Score (NES). Regulons with a high confidence score of A–B were analysed, and cases with $p < 0.05$ NES of ± 2 were considered significantly enriched.

5.4 MS monocytes, mDCs and tolDCs share alterations in the AHR pathway

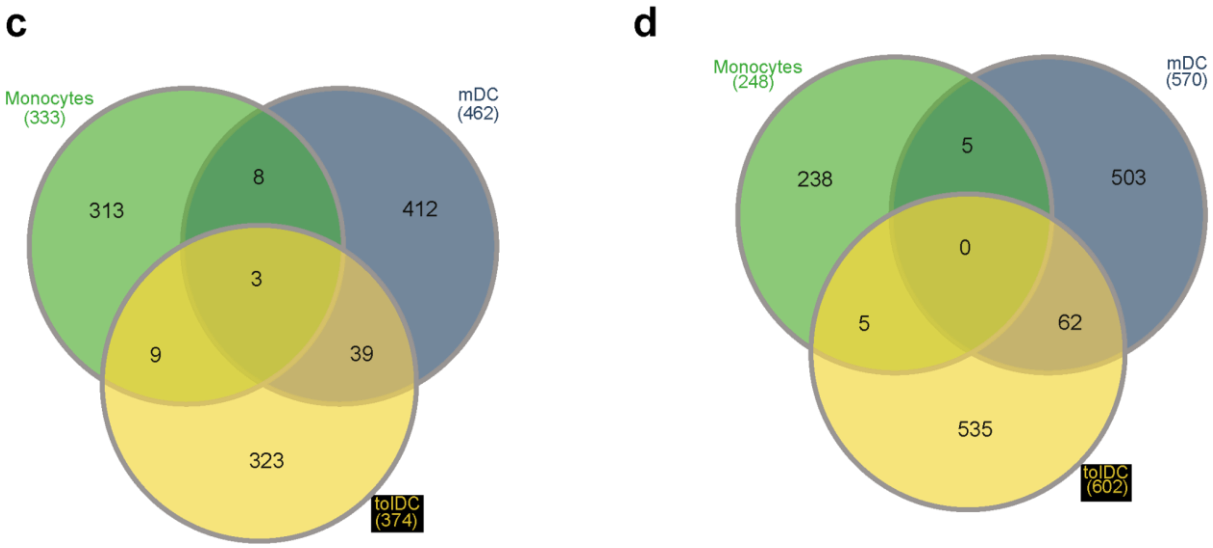
To identify pathways that are altered in MS Mono and whose dysregulation persists across the *in vitro* differentiation to MS mDCs or MS tolDCs, weinspected common DMPs and DEGs across the three different cell types. In relation to DNA methylation (Fig.4a), after annotating DMPs to the single nearest gene, we found that only one differentially methylated gene was shared across the three cell types, annotating to *AHRR*.



(4a) Venn diagram showing shared hyper and hypomethylated genes linked to significant differential methylation changes (DMPs) across HD-MS contrasts, in different cell types (MS

Mono, MS mDCs and MS tolDCs). **(4b)** Violin plots showing DNA methylation levels (*b*-values) of AHRR individual CpGs in hypermethylated and hypomethylated sets across all three comparisons. P-values correspond to the false discovery rate (significant if FDR < 0.05) calculated in the limma package.

In relation to the occurrence of common transcriptomic alterations, MS monocytes, mDCs and tolDCs shared upregulation of *PPBP*, which is associated with positive regulation of immunity (290). In addition, *MSLN* and *PKHD1L1* were upregulated, although their roles in innate immunity is not known (Fig. 4c). On the other hand, no shared differentially downregulated genes were found across the three cell types (Fig. 4d).

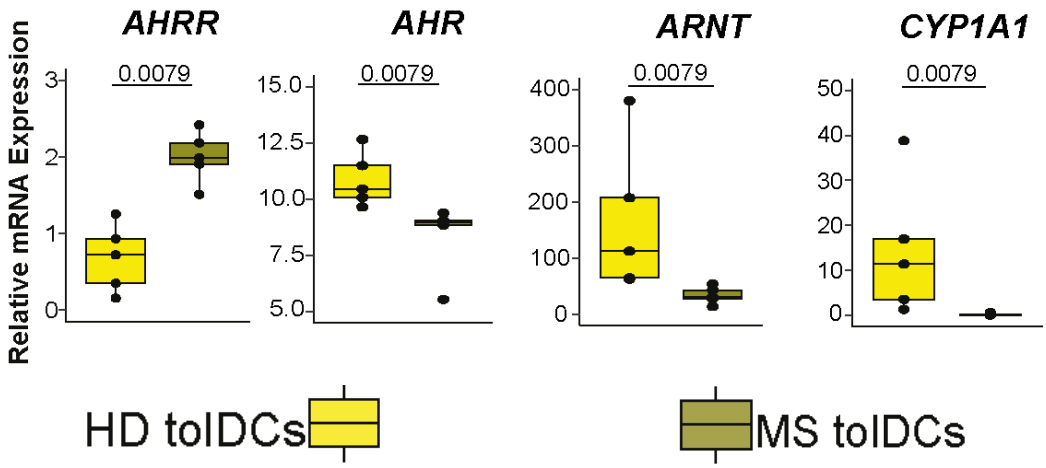


(4c) & **(4d)** Venn diagram showing shared differentially upregulated (**a**) and downregulated (**b**) genes across MS Mono, MS mDCs and MS tolDCs.

In addition to the aforementioned changes in *AHRR* methylation levels, MS monocytes showed increased expression of *AHRR*, while MS tolDCs had reduced expression levels of *CYP1A2* in the RNAseq dataset, suggesting the occurrence of alterations of the AHR pathway in MS monocytes and derived cells. To validate this in MS tolDCs, we quantitated the transcript levels of *AHRR*, *ARNT*, *AHR*, and *CYP1A1* in tolDCs from two additional cohorts of MS

patients and HD. *ARNT encodes* the AhR translocator protein and is also known as *HIF1b and CYP1A1 is* an AHR target that can then be used as a surrogate of AHR activity. MS tolDCs showed higher mRNA levels of *AHRR* and lower levels of *ARNT* and *AHR* (Fig. 4e). In line with this, *CYP1A1* expression was markedly higher in HD tolDCs than in MS tolDCs (Fig. 4e).

e



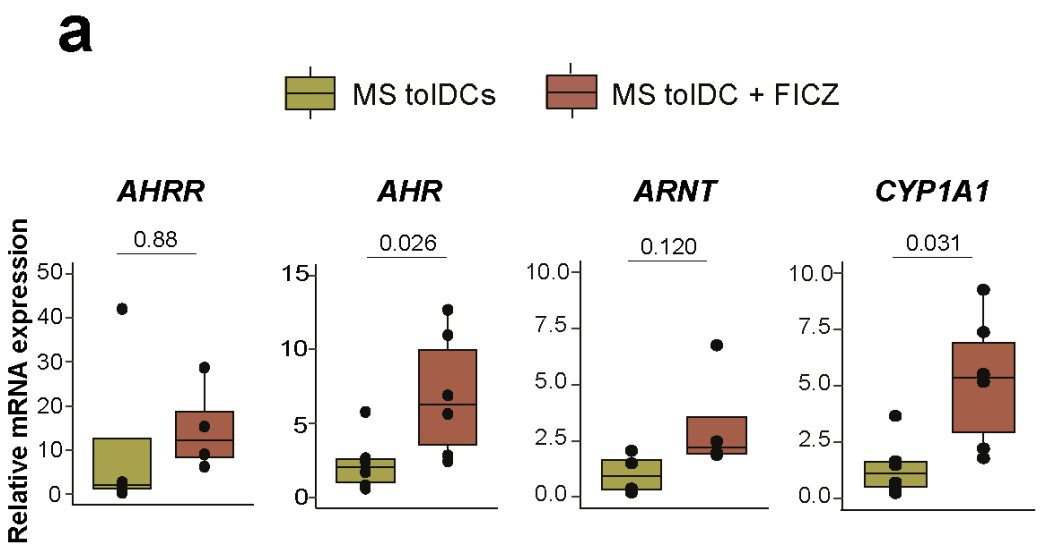
(4e) Box plot of relative expression of individual genes performed by RT-qPCR of mRNA in HD tolDCs vs MS tolDCs. *P*-values from Wilcoxon tests are shown.

Overall, the AHR pathway was dysregulated in MS tolDCs at the level of gene expression and DNA methylation.

5.5 Modulation of the AHR pathway influences the tolDC functonal profile

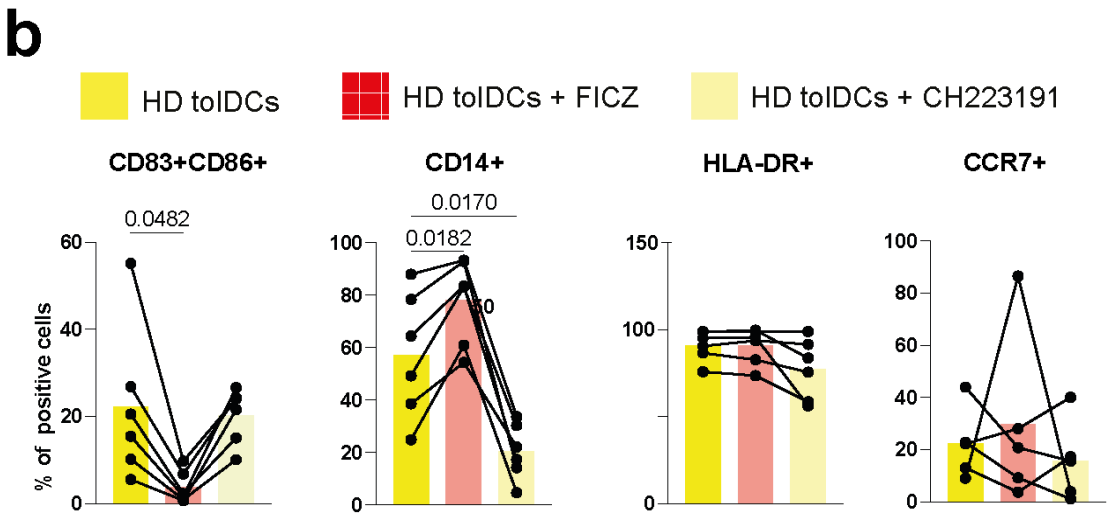
To prove that AHR is implicated in the acquisition of the tolerogenic program of our cell therapy, we differentiated VitD3 tolDCs in the presence of a specific agonist (FICZ) or an inhibitor (CH223191) of AHR and evaluated their effects

on gene expression and functionality. First, the AHR agonist FICZ induced increased expression of the *AHR* gene and *CYP1A1* in MS tolDCs, supporting the occurrence of activation of the pathway (Fig. 5a).



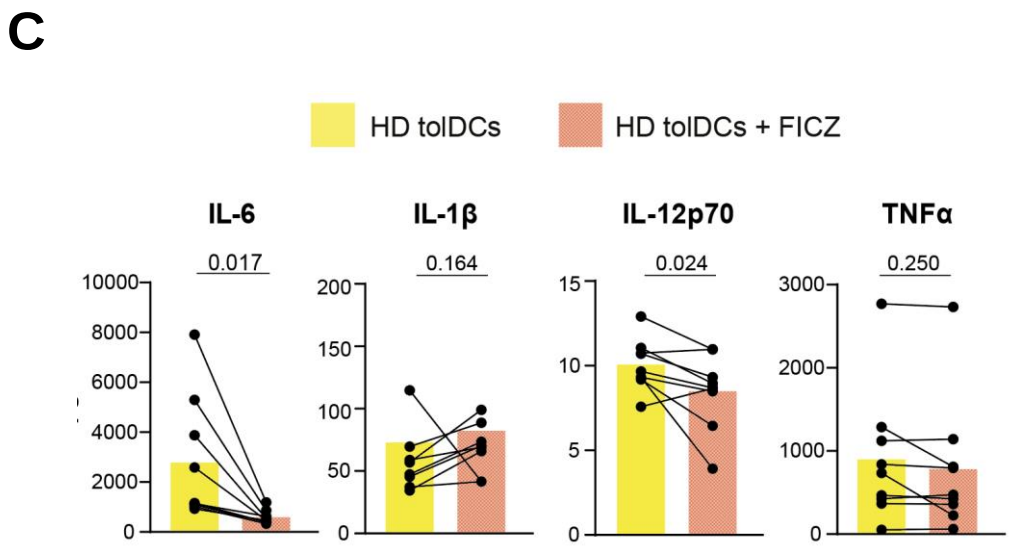
(5a) Box plot of relative expression of individual genes performed by RT-qPCR of mRNA in MS tolDCs versus MS tolDCs + FICZ. P-values from Mann-Whitney tests are shown.

On the other hand, FICZ agonism did not induce any significant change in the expression of *AHRR*, and *ARNT*. AHR agonism with FICZ increased expression of CD14 and downregulated CD83 and CD86, while antagonism with CH223191 caused reduction of CD14 expression (Fig. 5b). No significant changes in HLA-DR and CCR7 were observed using the agonist or antagonist. Overall, the AHR agonist induced a more immature and tolerogenic phenotype, while the opposite is observed with AHR antagonism.



(5b) Before-after Scatter Bar plot showing flow cytometry data relative to the percentage of CD83CD86, CD14, CCR7 or HLA-DR positive cells among tolDCs, tolDCs + FICZ and tolDCs + CH223191. P-values from ANOVA with multiple comparisons are shown.

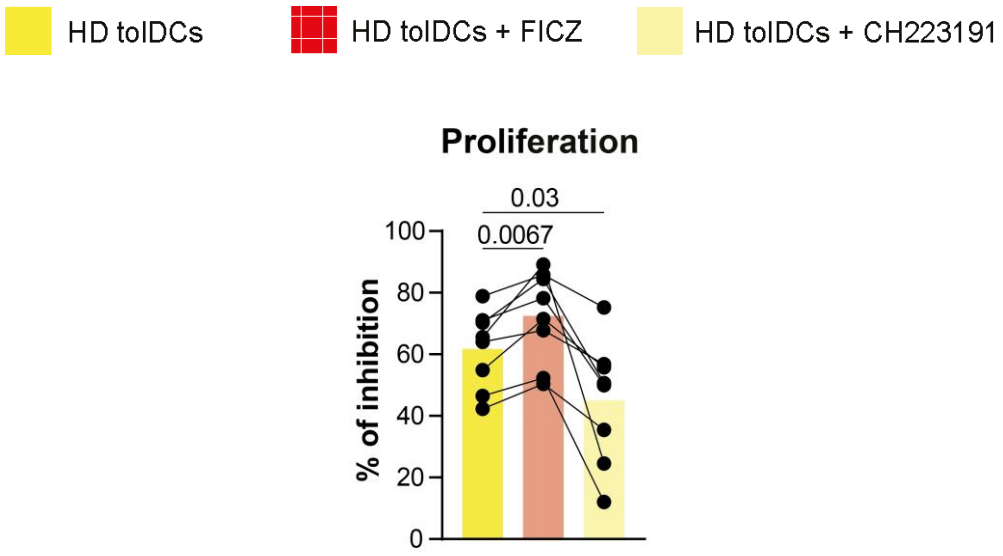
In addition, HD tolDCs differentiated with FICZ produced less IL-6 and IL-12p70, while no differences were observed in IL-1β and TNF-α production (Fig. 5c).



(C) Before-after scatter Bar plot representing the effect of FICZ agonist on tolDCs production of IL-6, IL-12p70 and IL-1β cytokine production. FICZ was added at day 0 and day 4 of differentiation of tolDC, with a final concentration of 18uM. P-values from Wilcoxon t-tests are shown.

This effect is further supported by functional data obtained by allogeneic mixed lymphocyte reaction (MLR), in which HD tolDCs differentiated in the presence of FICZ were less able to induce allogeneic PBMCs proliferation in comparison to conventional tolDCs, while tolDCs differentiated in presence of CH223191 induced more proliferation (Fig. 5d).

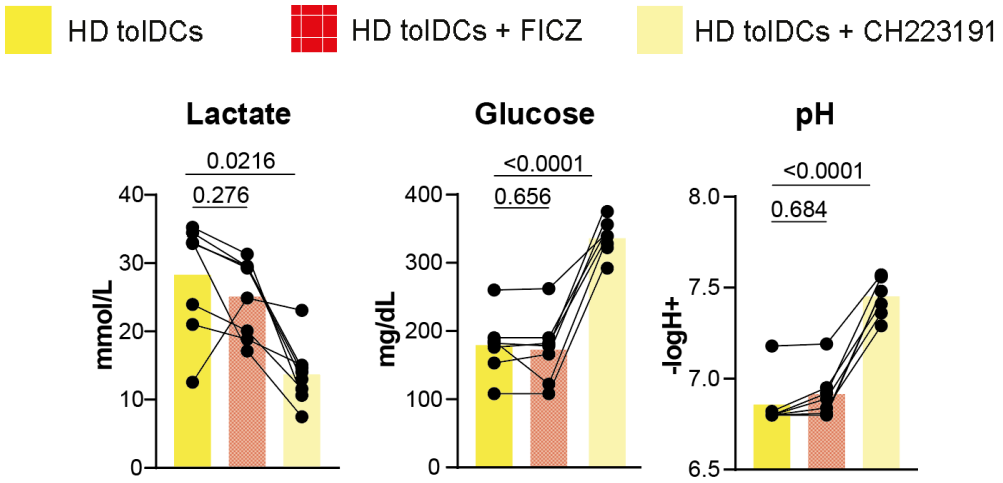
d



(5d) Proliferation of allogeneic peripheral mononuclear cells co-cultured with HD tolDCs, tolDCs differentiated either in the presence of FICZ (HD tolDC + FICZ) or CH223191 (HD tolDCs + CH223191). Inhibition of proliferation was assessed as the percentage of positive Violet 450 lymphocytes and calculated using mDC-induced proliferation as reference for each sample by using the following formula “mDCs-tolDCs/mDCs”, obtaining the percentage of reduction of proliferation of tolDCs condition in reference to the donor-matched mDC. P-values from ANOVA with multiple comparisons are shown.

Finally, the AHR antagonist showed direct effects on the metabolism of tolDCs, with an increase in the pH of the medium and a reduction in both glucose consumption and lactate production (Fig. 5e). Glycolysis is a hallmark of VitD3 tolDCs metabolism (291) and lactate plays an important role in defining their tolerogenic function (194,218).

e



(5e) Biochemical quantification of pH, glucose and lactate concentration on day 6 cell culture supernatants. P-values from ANOVA with multiple comparisons are shown.

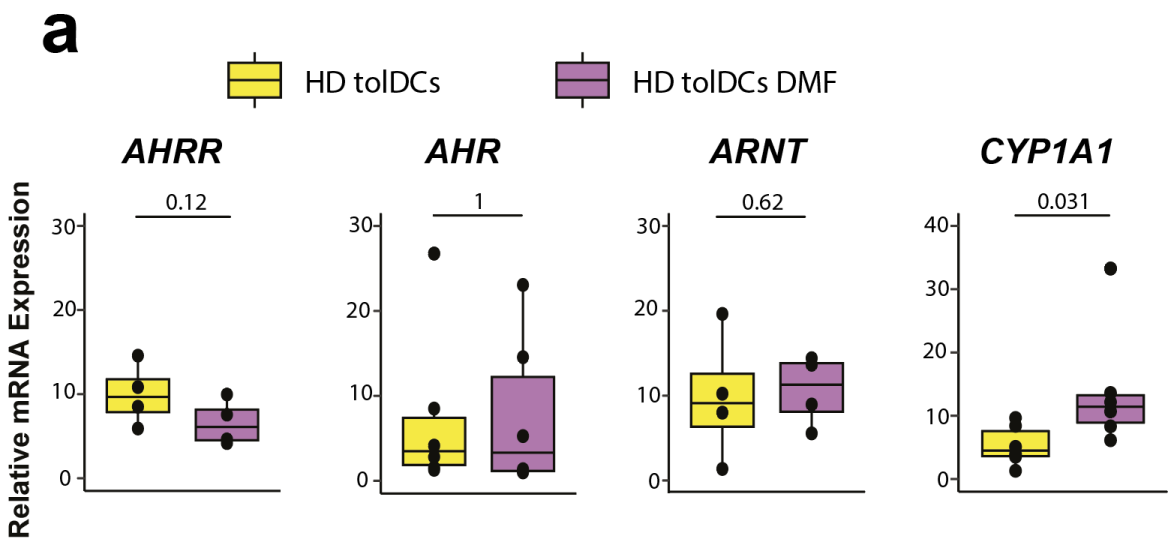
Taken together, these results led us to hypothesise that AHR is at least partially implicated in defining VitD3-tolDC functionality and that direct agonism of this pathway has a positive effect on it.

5.6 In vitro DMF supplementation boosts VitD3 tolDCs tolerogenicity

While the direct agonism of AHR with FICZ showed an improvement of the tolerogenic features of MS tolDCs, clinical administration of this molecule to MS patients is problematic due to its fast pharmacokinetic and instability (292). Moreover, FICZ can promote the differentiation of CD4 Th17 cells, which are important drivers of MS pathogenesis (293). On the other hand, dimethyl fumarate (DMF) is an oral fumaric acid ester already approved for the treatment of RRMS and active SPMS, that possesses immunomodulatory properties and a good tolerability profile.

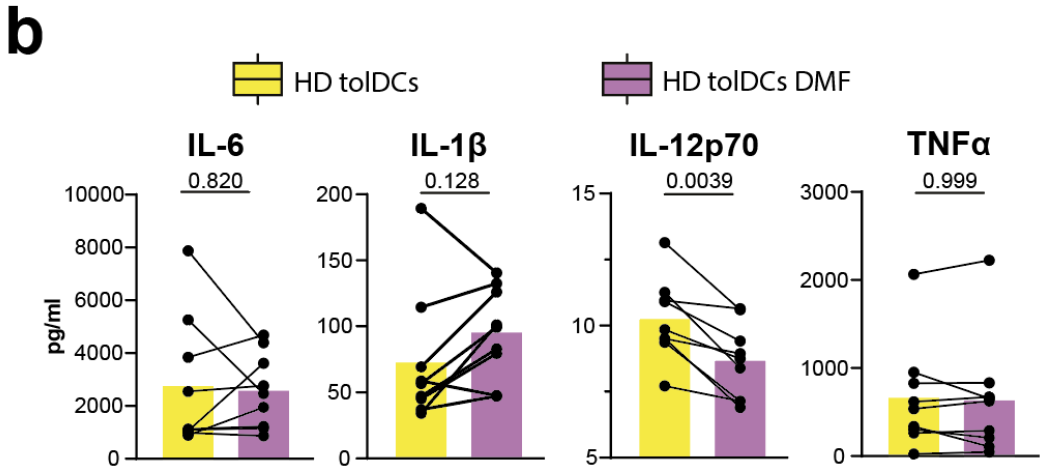
Specifically, DMF is a strong activator of NRF2 transcription factor and inhibitor of NFkB (294,295) and thus mimics through these mechanisms the signature of AHR-agonism in myeloid cells.

Moreover, DMF seems able to upregulate the AHR signature directly or indirectly through NRF2 (296,297)v.For these reasons, we explored the effects of DMF on tolDCs gene expression, metabolism and functionality, aiming to identify a surrogate for an AHR agonist and to decrease NFkB signalling. First, as a proof of concept, we checked the effect of DMF along the differentiation from HD monocytes to HD tolDCs. Analysis of qPCR data showed that DMF triggers *CYP1A1* expression, while *AHR, AHRR* and *ARNT* transcript levels do not change (Fig. 6a).



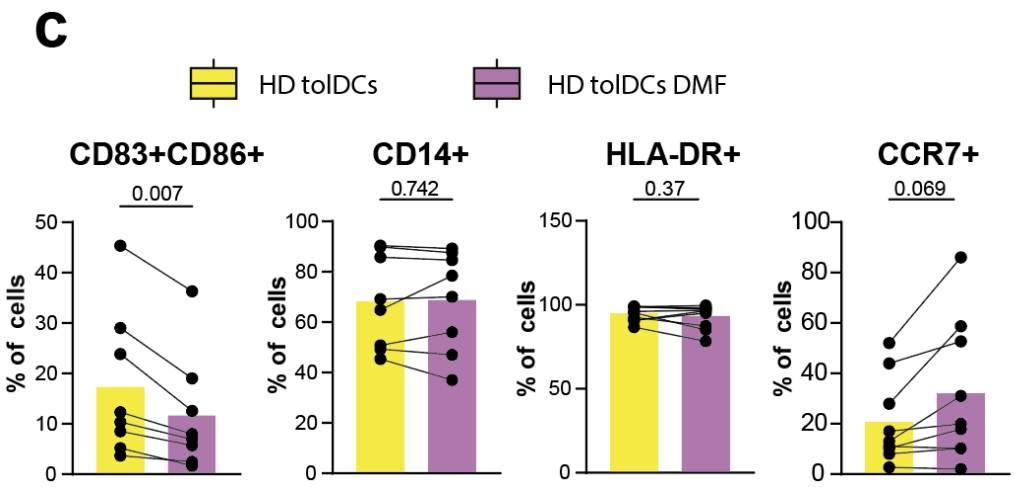
(6a) Box plot of relative expression of individual genes performed by RT-qPCR of mRNA in HD tolDCs vs HD tolDCs + DMF. DMF was added at day 0 and day 4 of differentiation of tolDCs, with a final concentration of 10uM. *P*-values from Wilcoxon tests are shown.

From a functional point of view, DMF *in vitro*-treated HD tolDCs (HD tolDCs DMF) produced less IL-12p70 in comparison to HD tolDCs (Fig. 6b), suggesting a less immunogenic phenotype.



(6b) Before-after Scatter Bar plot representing the effect of DMF on tolDCs production of IL-6, IL-12p70 and IL-1β cytokine production. tolDCs HD shows data already presented in Fig 3f. *P*-values from Wilcoxon tests are shown.

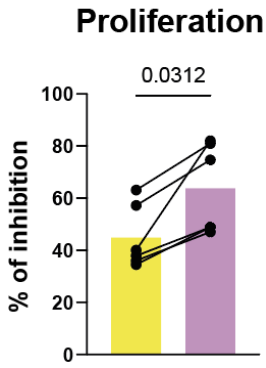
Flow cytometry data show that HD tolDCs DMF express less costimulatory molecules CD83 and CD86 (Fig. 6c). No effects were observed on CD14 and HLA-DR expression (Fig. 6c).



(6c) (C) Before-after Scatter Bar plot showing flow cytometry data relative to the percentage of CD83CD86, CD14, HLA-DR and CCR7 positive cells among HD tolDCs and HD tolDCs DMF. *P*-values from Wilcoxon tests are shown.

Importantly, HD tolDCs DMF inhibited more allogeneic proliferation in allogeneic MLR in comparison to HD (Fig. 6d).

d



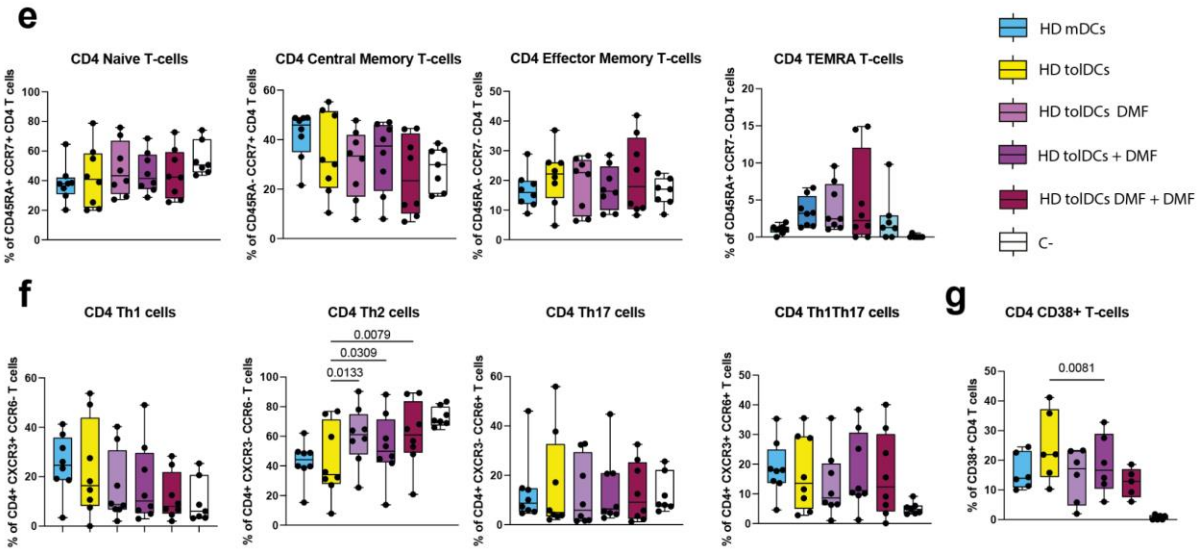
(6d) Proliferation of allogeneic peripheral mononuclear cells co-cultured with HD tolDCs and HD tolDCs DMF. Inhibition of proliferation was assessed as described before. *P*-values from Wilcoxon tests are shown. Yellow bars represent HD tolDCs and pink bars HD tolDCs + DMF.

Overall, in vitro DMF treatment with DMF was able to induce a stronger tolerogenic functionality in tolDCs, which was only partially recapitulative of direct FICZ agonism.

Finally, we studied T cell polarisation after healthy donor-derived DC-PBMC co-cultures in different experimental conditions: tolDCs alone (HD tolDCs), tolDCs differentiated in the presence of DMF (HD tolDCs DMF), tolDCs alone in which 10uM DMF is added in the DC-PBMC co-culture (HD tolDCs + DMF), tolDCs differentiated in presence of DMF and in which 10uM is added to the DC-PBMC co-culture (HD tolDCs DMF + DMF) and only PBMC without any stimuli as negative control (C-). After 6 days of coculture, no differences were observed in the percentages of naive, central memory, effector memory or terminally

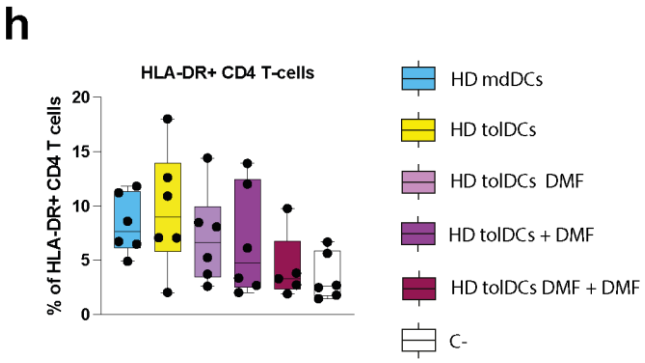
differentiated effector memory (TEMRA) CD4 T cells among the different groups (Fig. 6e).

On the other hand, there was an increase in the percentage of CD4 T-helper type 2 (Th2) in cocultures with HD tolDCs DMF + DMF in comparison to the other groups (Fig. 6f) and lower expression of the activation marker CD38 (Fig. 6g) in total CD4 T cells.



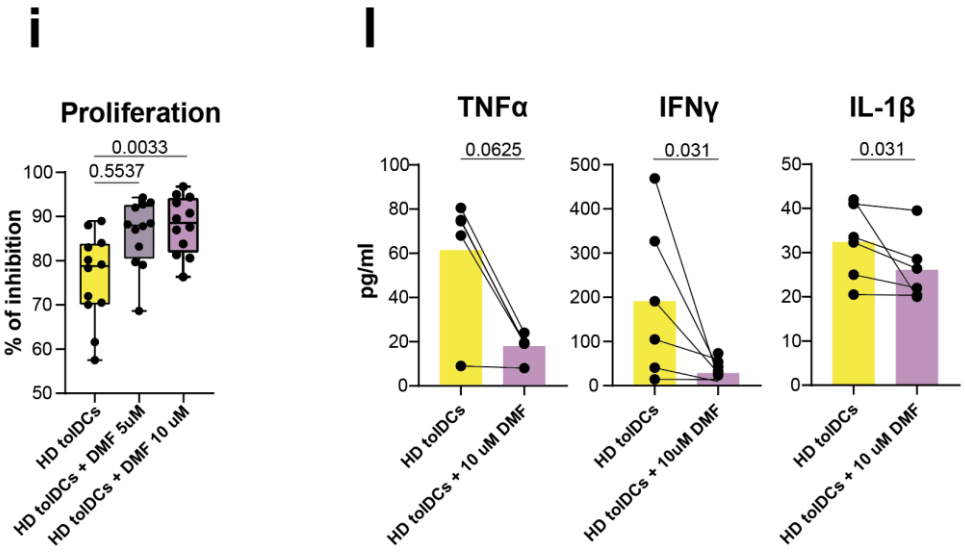
(6e) Box plots of percentage of positive CD4 T cells: Naive (CD45RA+ CCR7+), Central Memory (CD45RA- CCR7+), Effector Memory (CD45RA- CCR7-) and Terminally-differentiated Effector Memory T-cells (TEMRA, CD45RA+ CCR7-) analyzed through flow cytometry after 6 days of DCs-PBMCs allogeneic co-cultures. P-values (p) from ANOVA with multiple comparisons are shown (Mixed-effects analysis). Different co-culture condition involve PBMCs together with HD mDCs, HD tolDCs, HD tolDCs differentiated in the presence of DMF (HD tolDCs DMF), HD tolDCs where DMF is added directly in the coculture (HD tolDCs + DMF), HD tolDCs differentiated in presence of DMF and for which DMF is added directly in the coculture (HD tolDCs DMF + DMF) and no tolDCs (C-). **(6f)** Box plots of the percentage of positive CD4 Th1 (CXCR3+ CCR6-), Th2 (CXCR3- CCR6-), Th17 (CXCR3- CCR6+) and Th1Th17 (CXCR3+ CCR6+) T-cells analysed through flow cytometry after 6 days of DCs-PBMCs allogeneic co-cultures. P-values (p) from ANOVA with multiple comparisons are shown (Mixed-effects analysis). Coculture conditions are the same as in Fig. 6e. **(6g)** Box plots of the percentage of positive total CD4 T-cells expressing the activation marker CD38 after 6 days of DCs-PBMCs allogeneic co-cultures. P-values (p) from ANOVA with multiple comparisons are shown (Mixed-effects analysis). Coculture conditions are the same as in Fig. 6e.

Instead, HLA-DR expression was not affected (Fig.6h).



(6h) Percentage of total CD4+ HLA-DR+ T-cells after 6 days DC-PBMC cocultures. P-values from ANOVA with multiple comparisons (Mixed-effects analysis) are shown in case of statistical significance.

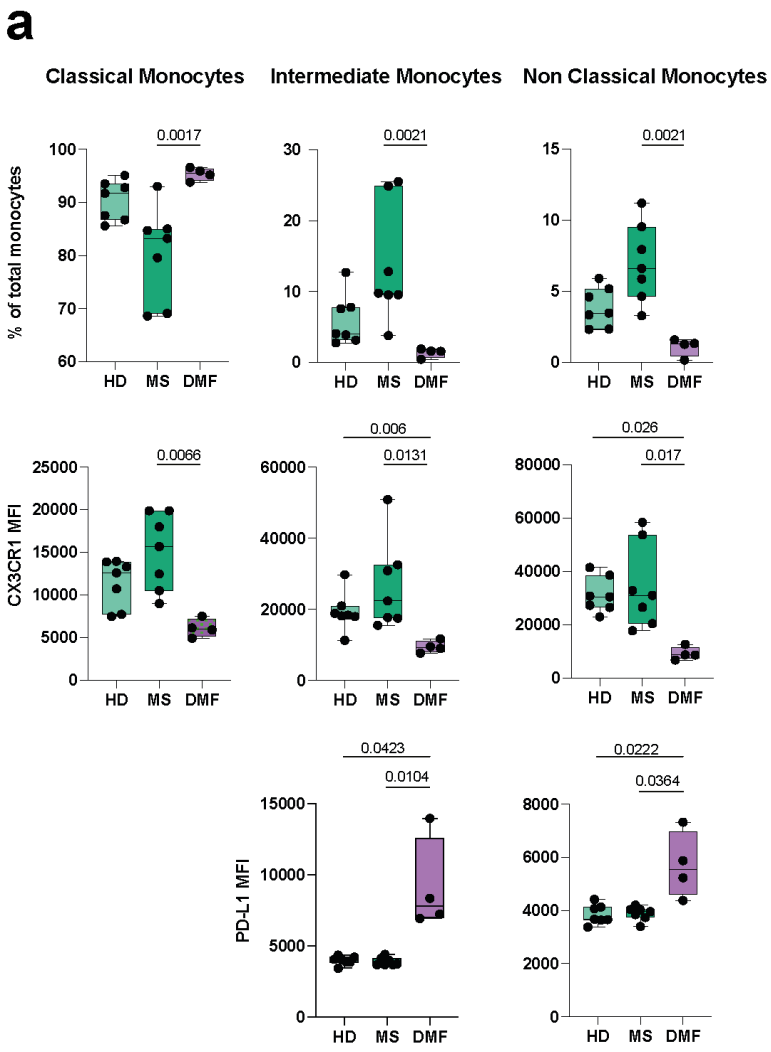
Finally, the direct addition of 10 uM DMF to tolDC-PBMC allogeneic MLRs, but not of 5uM DMF, determined less proliferation and concomitant reduction in IFNγ and IL-1β production in comparison to HD tolDC alone (Fig. 6h and Fig. 6i).



(6i) Proliferation of allogeneic peripheral blood mononuclear cells co cultured with HD tolDCs without or with the presence of DMF 5uM or 10 uM (HD tolDCs + 5uM or 10uM DMF), in a 1:10 DC-PBMC ratio. Inhibition of proliferation was assessed as described before. One-way ANOVA with multiple comparisons was used to calculate significant differences among groups, reported as P values. **(6l)** Before-after Scatter Bar plot representing the effect of DMF on tolDCs production of IL-6, IL-12p70 and IL-1β cytokine production. DMF was added during the coculture with HD tolDCs and allogeneic PBMCs at day 0 (HD tolDCs + 10uM). P-values from Wilcoxon tests are shown.

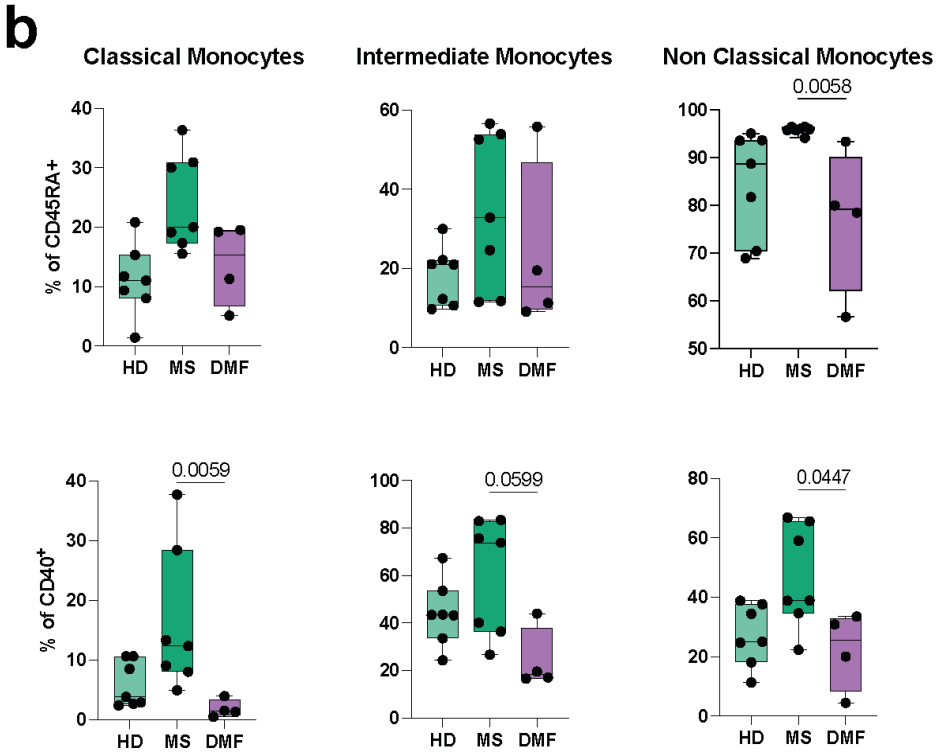
5.7 In vivo DMF administration to MS patients restores fully functional tolDCs

Then, we evaluated whether *in vivo* administration of DMF to MS patients could influence the functionality of MS tolDCs. First, we profiled through spectral flow cytometry the expression of markers in monocytes from a new cohort of MS patients receiving DMF treatment (MS DMF) and the previous cohorts of HD and naive MS patients (MS). Similarly to HD, MS DMF patients showed higher percentages of classical monocytes and less intermediate and non-classical monocytes in comparison to MS patients (Fig. 7a, first row).



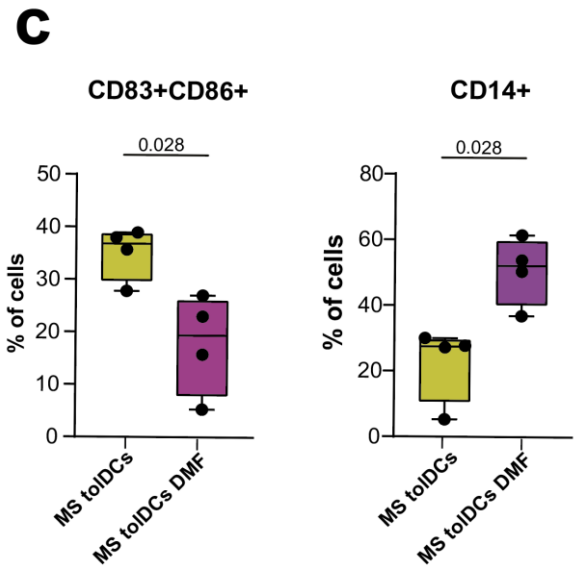
(7a) Boxplots reporting percentages of Classical (CD14++ CD16-), Intermediate (CD14+ CD16+) and Non-classical (CD14+ CD16++) monocytes among HD and MS patients without treatment (MS) or treated with Dimethyl Fumarate (DMF), with respect to total monocytes as parent gate (First row), or reporting the Median fluorescence intensity (MFI) of CX3CR1 (second row) or PD-L1 (third row) positive cells with respect to Classical, Intermediate or Non-Classical monocytes. *P*-values from Mann-Whitney test are shown in case of statistical significance. Percentages of Classical, non-classical and intermediate monocytes from HD and MS patients have already been shown in Fig.1b but new statistical tests have been done to include DMF-treated patients.

Moreover, in comparison to MS patients, classical, intermediate and non-classical monocytes from MS DMF patients showed lower expression of CX3CR1 (Fig. 7a, second row), a chemokine receptor involved in trafficking to inflammation sites and the CNS in MS (298). DMF also induced higher expression of PD-L1 in intermediate and non-classical monocytes in comparison to MS patients and HD (Fig. 7a, third row). On the other hand, expression of PD-L1 was not detected in classical monocytes (data not shown). Finally, DMF treatment reduced the expression of CD45RA in non-classical monocytes and of CD40 in classical and non-classical subsets (Fig. 7b).



(7b) Boxplots reporting percentages of CD45RA (first row) or CD40+ (second row) Classical (CD14++ CD16-), Intermediate (CD14+ CD16+) and Non-classical (CD14+ CD16++) monocytes among HD, MS patients without treatment (MS) or patients treated with Dimethyl Fumarate (DMF). *P*-values from one way ANOVA (Kruskal Wallis test) with multiple comparisons are shown. Percentages of CD45RA and CD40+ Classical, non classical and intermediate monocytes from HD and MS patients groups have already been shown in Fig.1b but new statistical tests have been done to include a new cohort of DMF-treated patients.

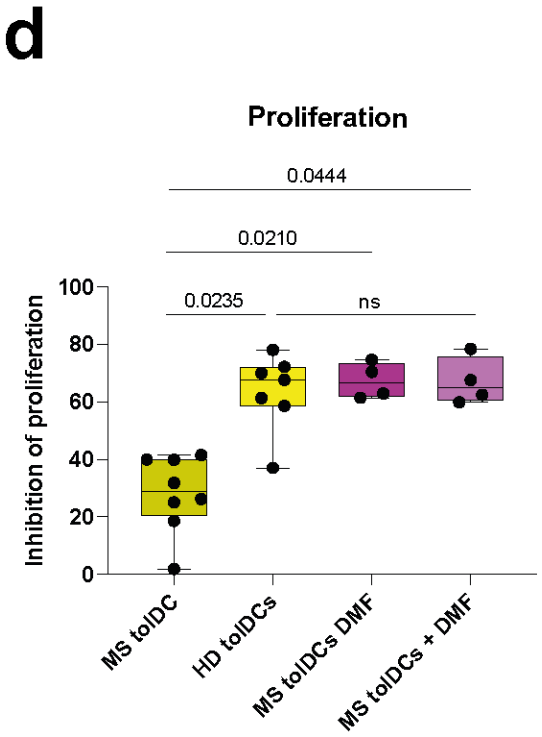
Secondly, we differentiated tolDCs from monocytes obtained from naive patients (MS tolDCs) and from patients receiving DMF treatment for at least 6 months (MS tolDC DMF) and compared their phenotype at day 6 of culture via flow cytometry. MS tolDCs DMF are characterised by a higher expression of CD14 and a decreased double positive CD83 CD86 population (Fig. 7c).



(7c) Box plot showing flow cytometry data relative to the percentage of CD83+CD86+ and CD14+ cells in MS tolDCs and tolDCs isolated from patients undergoing DMF treatment (MS tolDCs DMF) at the end of the 6-days *in vitro* differentiation protocol. P-values were calculated through Mann-Whitney tests.

Then, to define the effect of in vivo DMF administration on the functionality of tolDCs, we studied through allogeneic MLR tolDCs differentiated from HD, MS patients (MS tolDCs), MS patients receiving DMF treatment (MS tolDCs DMF) and MS patients in which DMF was added *in vitro* during the differentiation (MS

tolDCs + DMF). MS tolDCs suppressed less allogeneic PBMCs proliferation in comparison to HD tolDCs, as also to MS tolDCs DMF and MS tolDCs + DMF (Fig. 7d). On the other hand, MS tolDCs DMF and MS tolDC + DMF showed an inhibition of allogeneic proliferation that was comparable to the one of HD (Fig. 7d).

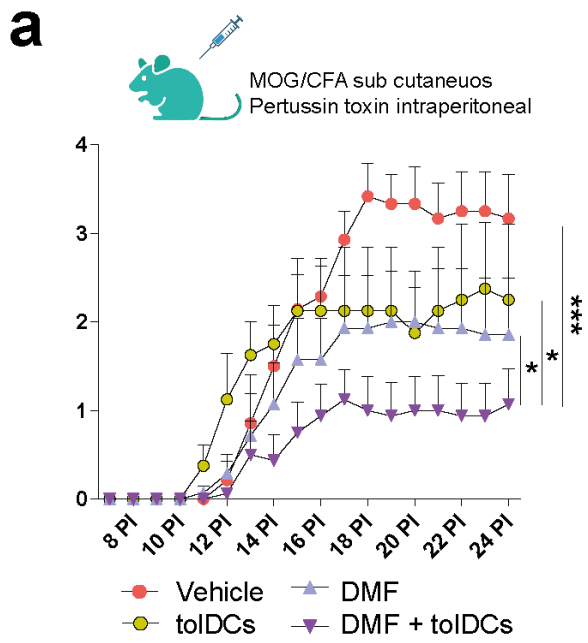


(7d) Proliferation of allogeneic peripheral mononuclear cells co cultured with tolDC from HD, treatment-naive MS patients (MS tolDCs), tolDCs isolated from patients undergoing DMF treatment (MS tolDCs DMF) or tolDC from active/naive MS patients differentiated in presence of DMF *in vitro* (MS tolDCs + DMF), in a 1:10 DC-PBMC ratio. Inhibition of proliferation was assessed as described before. One-way ANOVA with multiple comparisons was used to calculate significant differences among groups, reported as P-values.

5.8 Combined therapy with DMF and tolDCs has higher clinical potential in comparison to monotherapies

Finally, we assessed the potential beneficial effects of a combined therapy of DMF + tolDC in the EAE model. To this end, we immunised C57Bl/6 mice with Myelin Oligodendrocyte Glycoprotein (MOG) 35-55 peptide and compared EAE mice treated with either a vehicle, DMF, bone-marrow-derived tolDCs loaded with MOG35-55 or with the combination of DMF and bone-marrow-derived tolDCs loaded with MOG35-55.

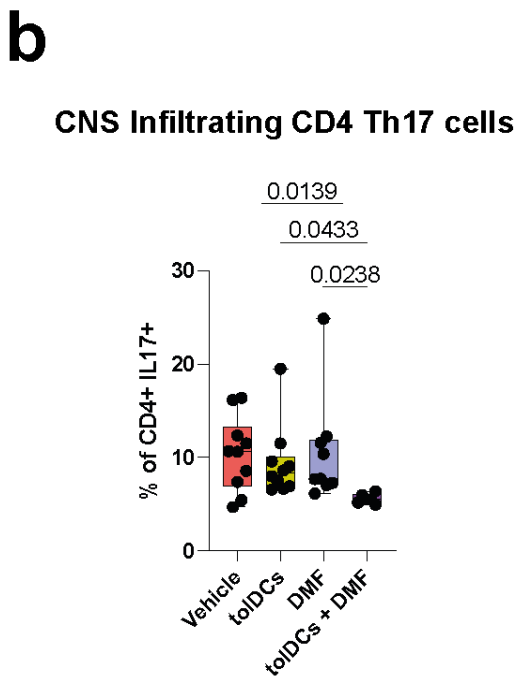
DMF + tolDCs treatment of EAE mice induced a significant reduction in the clinical score, in comparison to either DMF or tolDC monotherapies, which had a comparable effect (Fig. 8a).



(8a) Representation of daily mean clinical score of C57Bl/6 mice immunised with MOG35-55 peptide treated with vehicle (PBS) (red circle, n=7), Dimethyl Fumarate (DMF) (lavanda triangle, n=7), VitD3-tolDCs-MOG (tolDCs) (yellow circle, n=4) or VitD3-tolDCs-MOG+DMF

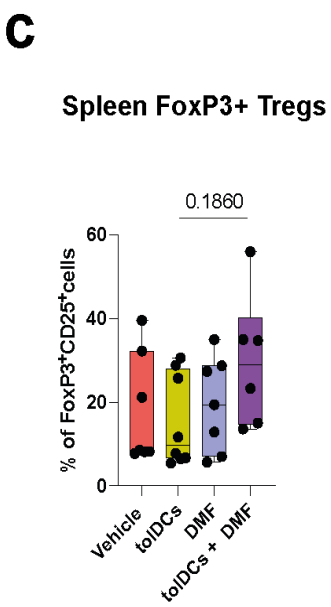
(tolDC+DMF, n=8) (purple inverse triangle) for 25 days of follow-up. P-values obtained from two-way ANOVA with multiple comparisons are shown (Holms-Sidak's multiple comparison test) (ns: P > 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001). Error bars represent mean ± SEM. All data from a single mouse experiment.

In addition, we isolated and analysed CD4 T cell infiltrates in mice spinal cords of the different treatment groups. Mice treated with the combined therapy showed a reduced infiltration of pathogenic IL-17-producing CD4 T cells in comparison to monotherapies (Fig. 8b), while no statistically significant differences were observed for IFNγ producing CD4 T cells (data not shown).



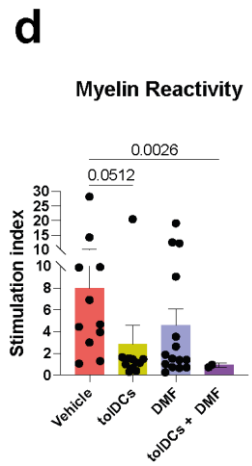
(8b) Boxplots showing the percentage of IL17+ CD4+ T cells in the cell infiltrate of spinal cords from mice treated with vehicle (PBS and methylcellulose, n=10), dimethyl fumarate (DMF, n=9), VitD3-tolDC-MOG (tolDCs, n=10) or VitD3-tolDCs-MOG + DMF (tolDCs + DMF, n=6) on day 24 pi. Samples were analysed through flow cytometry after intracellular and surface marker staining. P-values obtained from two-way ANOVA with multiple comparisons are shown (Kruskal-Wallis Test). Data from two independent experiments.

We then analysed the percentage of total CD25+ FoXP3+ Tregs present in mice spleens. However, statistical significance was not reached in any comparison between the different groups (Fig. 8c).



(8c) Boxplots showing the percentage of CD25+ FoxP3+ CD4+ T cells from mice treated with vehicle (PBS and methylcellulose, n=7), dimethyl fumarate (DMF, n=7), VitD3-tolDC-MOG (tolDCs, n=8) or VitD3-tolDCs-MOG + DMF (tolDCs + DMF, n=6) on day 24 pi. Samples were analysed through flow cytometry after intracellular and surface staining. *P*-values obtained from two-way ANOVA with multiple comparisons are shown (Kruskal-Wallis Test). Data from two independent experiments.

Finally, to evaluate if any of the therapies were able to induce tolerance of the immunising antigen, we stimulated EAE-derived spleens with MOG35-55 peptide for 4 days and checked splenocyte proliferation. Strikingly, we observed a reduction in MOG splenocyte reactivity in the combined therapy group versus vehicle and monotherapies, suggesting a stronger antigen-specific hyporeactivity against the autoantigen MOG (Fig. 8d).



(**8d**) Analysis of antigen-specific T cell reactivity to MOG35-55 in splenocytes from mice treated with vehicle (PBS and methylcellulose, n=11), dimethyl fumarate (DMF, n=15), VitD3-tolDC-MOG (tolDCs, n=11) or VitD3-tolDCs-MOG + DMF (tolDCs + DMF, n=8) on day 24 pi. The mean stimulation index was calculated for each group after 4 days of incubation. Error bars correspond to SEM. *P*-values obtained from one-way ANOVA with multiple comparisons are shown (Kruskal-Wallis Test). Data from two independent experiments.

6. Discussion

Since the FDA approval of the first therapeutic cellular products, hundreds of patients have benefited from cell therapies. In parallel, numerous clinical studies have assessed and continue to assess the use of cellular therapies in cancer, autoimmunity and transplantation. Indeed, cell therapies open important perspectives on how to treat, and possibly cure immune-mediated diseases. This is particularly important for autoimmune diseases like MS, in which the only available therapeutic options are not curative and involve lifelong immunosuppression. In this context, antigen-loaded tolDC-based therapies represent a possibility to re-educate the myelin autoreactive immune system of MS patients toward tolerance without causing general suppression of physiological immunity. However, autologous tolDCs are therapies generated from immune cells of patients with different grades of immune dysregulation, meaning that the starting material used to generate the final cell product could be carrying a pathogenic and/or inflammatory phenotype imprinted by the environment in which it originated and persisted. Indeed, an inflammatory-primed starting material could lead to DC therapies with suboptimal functionality, when compared to cells generated from healthy individuals. This idea is supported by several studies addressing the impact of the disease environment on cell therapy starting material characteristics in T cell immunotherapies for cancer treatment (299–302). However, the same type of studies has not been performed in the context of myeloid regulatory cell therapies, nor autoimmune diseases in general. However, the same type of studies has not been performed in the context of myeloid regulatory cell therapies, nor autoimmune diseases in general.

In our study, we tested this hypothesis in the context of MS patients and of a tolDC-based therapy for the first time. We first compared the phenotype of our starting material, CD14+ monocytes from MS patients and HD. Our results showed differences in terms of monocyte abundances, protein expression and transcriptomic and epigenomic signatures, which point towards an activated and proinflammatory monocyte state in MS versus HD. Firstly, MS Classical monocytes were reduced, while non-classical and intermediate monocytes were increased. Classical monocytes are cells able to secrete soluble mediators and to differentiate into monocyte-derived DC to regulate adaptive immune responses. On the other hand, intermediate monocytes are specialised in antigen presentation and are strong inducers of T cell proliferation and stimulation in inflammatory responses. Indeed, intermediate monocytes generally expand in inflammatory conditions (303) and produce high levels of TNF-α (304). This pro-inflammatory phenotype is further defined by the overexpression of inflammatory genes in the RNAseq database (*TNF, CCL4, IFNB1*), which were more clearly highlighted in the Dorothea analysis which showed direct enrichment of NFkB, STAT and Jun pathways.

Non-classical monocytes also increased. While this subset of monocytes can be anti-inflammatory, several studies (305) underline their pro-inflammatory and pathogenic role in MS and other autoimmune diseases. This consideration is supported by an enrichment of CD45RA+ and CD40+ classical and non-classical monocytes in MS. Indeed, CD45RA and CD40 are two activation markers whose expression has been already described during inflammation in different pathological settings (306,307). Moreover, we observed increased expression of the fractalkine receptor CX3CR1 in all three monocyte subsets in MS patients versus HD. CX3CR1, or fractalkine receptor, is a chemokine receptor involved in trafficking to the CNS and inflammation sites in MS (298), which could equip MS monocytes with increased migratory capacity to the brain.

DNA methylation also points out differences between MS and HD patients, which were mainly related to an enrichment of inflammatory factors (AP-1, Fos, JunB) that are also targets of NFkB (308), as of ontology categories linked to immune response. Taken together, our results offer proof of the existence of a pro-inflammatory, activated CD14+ fraction in MS patients, which is also confirmed by other studies describing various degrees of monocyte dysregulation at the transcriptomic and epigenomic levels in MS patients (309–313). We speculate that CNS inflammation, blood-brain-barrier disruption and elevated serum and CSF levels of proinflammatory cytokines could imprint MS monocytes with the pro-inflammatory phenotype that we observed. Overall, we also cannot exclude that these increased levels of non-classical and intermediate monocyte subpopulations could drive the enrichment in the inflammatory signature seen in our bulk transcriptomic and epigenomic data. In this context, studies focused on a single-cell approach could help to better identify which is the driver of this altered phenotypic state.

Transcriptomic and DNA methylation analysis of monocyte-derived mDCs and tolDCs pointed out that the proinflammatory signature of MS monocytes is conserved across *in vitro* differentiation, leading to DC with a phenotype enriched in inflammatory pathways. This is particularly true for mDCs, which show upregulation of mTOR at the RNA level and wide demethylation and enrichment in inflammatory factors as NFkB at the epigenomic and transcriptomic level, mimicking what was observed in MS monocytes. Enrichment of NFkB and mTOR in MS mDCs offers several new targets that could be therapeutically targeted with inhibitors to modulate *in vivo* immunogenic DCs in MS patients. The administration of mTOR inhibitors such as rapamycin to MS patients harbours therapeutic potential, given that it could contribute to tolerating hyperinflammatory DCs, T and B cells that are typical of this disease and contribute to its pathogenesis. Indeed, this enrichment of

canonical inflammatory pathways in MS mDCs allows us to hypothesise that this alteration is also naturally occurring in *vivo* monocyte-derived DC (mo-DC), which could present a proinflammatory, and even pathogenic, role in MS. While this idea is supported by several studies highlighting alterations of different subpopulations of DC (314–316)in MS patients and the EAE model, few studies address this aspect in mo-DC.

Interestingly, MS tolDC did not show wide methylation changes as the one observed in MS mDCs. This can be explained by previous work from our group (206), highlighting the role of Vitamin D3 as an epigenetic remodeler, and could also indicate that our tolerising protocol involving Vitamin D3 is able to revert most of the aberrant epigenetic signature present in MS Mono and still maintained in MS mDCs. Despite this, MS tolDCs were less able to decrease proliferation in our allogeneic MLR experiments in comparison to HD, suggesting that reversion of DNA methylation by itself could not completely re-establish the full functionality of MS tolDCs. Indeed, MS tolDCs still show conspicuous differences in their transcriptomic profile, with a general overexpression of key markers linked to DC maturation, activation and immunogenicity (*CD1c, CD1a, CD40LG*) (317,318) and downregulation of *ARG1*, an important factor of the VitD3 tolDC gene program (319), which may partially influence this reduced suppressive capability.

Integration of our DNA methylation data exposed that MS Mono, MS mDC and MS tolDCs shared significant demethylation changes in CpGs related to *AHRR*, the repressor of AHR. Moreover, *AHRR* expression was upregulated in MS mono RNAseq and *CYP1A2*, a surrogate marker of AHR activity, was downregulated in MS tolDCs. Further validation of gene expression through qPCR in MS tolDCs confirmed downregulation of AHR program key genes. This is in line with studies highlighting systemic alteration of the AHR pathway in MS and its correlation with clinical features (320,321).

At the cellular level, AHR can imprint either pro- or anti-inflammatory features in the T cell compartment according to the type of agonist and immunological context (293,322), its activity in DC is linked to the acquisition of tolerogenic features. Interestingly, a recent study performed on DC-10, a type of mo-tolDC cell differentiated in the presence of IL-10 as a tolerising factor, highlighted AHR as a master regulator of the DC10 program in a mechanism involving autocrine IL-10 signalling (251). Moreover, this same study showed that MS DC-10 are functionally defective in comparison to the ones differentiated from HD, supporting our findings.

In its relapsing-remitting phase, MS is a prominently inflammatory disease in which both adaptive and myeloid immunity are hyperactivated. In this scenario, immune cells are activated and physiological tolerogenic mechanisms are not able to “tolerise” them as efficiently as in healthy individuals. Moreover, MS patients show various grades of dysregulation of central and peripheral tolerance, exacerbating this phenomenon (323). In this context, AHR agonism could overcome MS-intrinsic defects in this signalling pathway, leading to fully functional DCs. Indeed, several studies showed that AHR agonism with FICZ, ITE (2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester) or other molecules can induce tolDCs *in vitro*, as clinical amelioration in EAE mice models (288,324–332).

While direct agonism of AHR with FICZ showed improvement of tolDC functionality in our dataset, real-world administration of this molecule to MS patients is problematic due to its fast pharmacokinetic and low stability (292). In this context, administration of the synthetic AhR agonist Laquinimod™ in EAE mice induced both amelioration of clinical score and induction of DCs with tolerogenic features through AHR agonism and NFkB downregulation (333,334). However, despite the encouraging preclinical data, clinical trials

(NCT02284568, NCT01707992) (335,336) investigating the use of Laquinimod in MS patients did not reach their primary endpoints, and higher doses were characterised by high toxicity of the treatment.

On the other hand, DMF is a methyl ester of fumaric acid already approved as first-line treatment in MS, whose principal mechanism of action involves activation of the transcription factor NRF2 and inhibition of NFkB (294,295). DMF acts on monocytes and can induce anti-inflammatory monocytes (337). Also, monocytes are among the main drivers of good response to DMF treatment in MS patients (131). In our study, DMF-treated patients show higher classical monocytes and lower levels of intermediate and non-classical monocytes in comparison to both HD and MS patients. Moreover, non-classical monocytes from MS DMF patients expressed less CD45RA, while both classical and non-classical monocytes expressed less CD40 in comparison to MS patients. DMF treatment induced higher expression of the immune checkpoint marker PD-L1 in intermediate and non-classical monocytes and lower CX3CR1 expression across all three subtypes in comparison to MS patients. Taken together, MS DMF monocytes appear to have a more regulatory profile in comparison to proinflammatory monocytes encountered in treatment-naive MS patients, at least according to the few markers analysed in this study.

Interestingly, reports suggest both direct and indirect interactions between AHR and NRF2, and both transcription factors can induce a tolerogenic signature in DCs by sharing several targets (296,297). Treatment of DCs with DMF, monomethyl fumarate (MMF) or other fumarates causes a reduction in the expression of costimulatory and maturation markers (140,338), and *in vivo* DMF treatment can induce IL-10-producing DC in humans (339). Our data confirmed the *in vitro* effect of DMF and showed that it has a synergic effect together with VitD3 in inducing tolDCs with reduced costimulatory molecule expression, pro-

inflammatory cytokine production and inhibition of allogeneic proliferation. Overall, except for IL-6 production and CD14 expression in HD tolDCs DMF only, DMF had a similar effect to FICZ when added *in vitro* to tolDCs, and was able to increase CYP1A1 activity, leading us to hypothesise an effect on AHR activity, as already described (296). However, while we were able to prove that DMF can induce CYP1A1 and possibly AHR activity, neither direct interaction of NRF2 and AHR, nor direct action of DMF on AHR has been mechanistically proved in our study, raising the need to better define the functional relationship between these factors.

Importantly, we showed for the first time that tolDCs differentiated from patients receiving DMF treatment had better tolerogenic functionality in comparison to the ones produced from naive patients, which was also comparable to the ones of healthy individuals. Moreover, this effect was also observed by adding *in vitro* DMF to MS tolDCs differentiated from naive patients, suggesting a direct involvement of DMF signalling in monocytes-to-tolDC differentiation. Given its capability to inhibit NFkB signalling and induce an AHR-like functional signature, DMF supplementation to MS patients or addition *ex vivo* during differentiation can revert MS Mono's pro-inflammatory signature, leading to more powerful tolDCs.

However, as supported by our results, an *in vivo* approach in which DMF is administered to MS patients before tolDCs generation and administration could offer more advantages. Indeed, *in vitro* addition of DMF to tolDCs allogeneic co-cultures induced less proliferation and, at the same time, lower production of IFNγ and IL-1β, suggesting modulation of both T cells and activated myeloid cells. IL-1/IL-1R1 signalling plays a crucial role in the onset and progression of MS by driving autoimmunity and neuroinflammation-induced damage in the CNS. To the best of our knowledge, the administration of IL-1 inhibitors has never been explored thoroughly in MS. In this sense, clinical experimentation

with IL-1/IL-R1 antagonists represents an important approach to explore in MS. Indeed, both TNF and IL-1b are downstream of NFkB positive regulation, which is strongly inhibited by DMF. This is supported by our data in which the combined therapy reduced IL-1β production and CD38 expression and showed a trend in reducing TNFα in *vitro* MLRs.

Finally, a DMF + tolDCs combined therapy was able to significantly ameliorate the clinical score in EAE. This reduction in disease severity was accompanied by reduced infiltration of CD4 Th17 T cells in the CNS, and a reduction of splenocyte reactivity to myelin antigens, suggesting induction of autoantigen hyporesponsiveness. While these results constitute preclinical proof of concept of this combined therapy, information regarding different functional profiles of BMDCs derived from healthy versus EAE mice which could corroborate our results in humans was not investigated in this study.

Given our results with the EAE model, we propose a combined therapy approach, in which simultaneous treatment in vivo/in vitro with DMF and tolDCs would exert both synergic and independent effects: on one side the beneficial immunomodulatory of DMF, reducing inflammation, T cell activation, and imprinting monocytes with a regulatory phenotype, boosting the functionality of tolDCs differentiated from the patient, and on the other side *in vitro* supplementation of DMF to tolDCs during the differentiation, which will lead to the generation of fully potent VitD3 tolDCs with maximal tolerance induction capability against autoreactive clones in MS and other autoimmune diseases.

7. Conclusions

The experimental evidence obtained from this thesis shows that:

1) Monocytes isolated from disease-active, treatment-naive, relapsing-remitting MS patients display alterations in subsets proportions and a distinct proinflammatory phenotype, as shown by methylomic and transcriptomic profiling, in comparison to those from HD.

2) The proinflammatory phenotype determined in MS monocytes is conserved across in vitro differentiation to mDCs and tolDCs, with the latter being characterised by reduced tolerogenic functionality.

3) MS monocytes and monocyte-derived mDCs and tolDCs share alterations in the AhR pathway at the DNA methylome and transcriptomic level.

4) In vitro direct agonism of AhR induces a stronger tolerogenic phenotype in monocyte-derived tolDCs, reverting MS-specific reduced tolerogenic functionality.

5) In vitro administration of the drug Dimethyl Fumarate mimics AhR agonism and produces MS tolDCs with increased tolerogenic properties.

6) In vivo administration of DMF to MS patients restores HD-like monocyte subpopulations and increases their expression of regulatory markers.

7) tolDCs differentiated from MS patients receiving DMF treatment have a higher suppressive capacity in comparison to naive MS patients, similarly to the one of tolDCs differentiated from HD monocytes

8) A combined therapy of DMF and tolDCs reduces the clinical score of mice with EAE in comparison to those treated with monotherapies

9) DMF + tolDCs combined treatment decreases Th17 CD4 T cell infiltration in EAE mice CNS and induce splenocytes myelin antigen hyporesponsiveness

Overall, this thesis shows the existence of specific phenotypic alterations affecting monocytes derived from MS patients, which determine the differentiation of MS tolDCs with reduced tolerogenic potency. In this sense, we conclude that the existence of intrinsic phenotypic modifications of innate immune cells in patients with autoimmune backgrounds and strong inflammatory status is an element to consider when designing successful myeloid regulatory cell therapies.

Moreover, we show that a multi-omic approach can lead to identifying these aforementioned degenerated pathways, which can be subsequently modulated to correct tolerogenic functionality.

Finally, this thesis strengthens the hypothesis that an approach aimed at both decreasing inflammation and inducing autoantigen-specific tolerance reeducation could offer increased therapeutic potential for RRMS patients in comparison to monotherapies.

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9. Publications

1) Català-Moll, F., Ferreté-Bonastre, A. G., Godoy-Tena, G., Morante-Palacios, O., Ciudad, L., Barberà, L., **Fondelli F.**, ... & Ballestar, E. (2022). Vitamin D receptor, STAT3, and TET2 cooperate to establish tolerogenesis. Cell reports, 38(3), 110244.

2) Mansilla, M. J., Presas-Rodríguez, S., Teniente-Serra, A., González-Larreategui, I., Quirant-Sánchez, B., **Fondelli, F.**, ... & Martínez-Cáceres, E. M. (2021). Paving the way towards an effective treatment for multiple sclerosis: advances in cell therapy. Cellular \& Molecular Immunology, 1-22.

3) Quirant-Sánchez, B., Mansilla, M. J., Navarro-Barriuso, J., Presas-Rodríguez, S., Teniente-Serra, A., **Fondelli, F.**, ... & Martínez-Cáceres, E. (2021). Combined Therapy of Vitamin D3-Tolerogenic Dendritic Cells and Interferon-β in a Preclinical Model of Multiple Sclerosis. Biomedicines, 9(12), 1758.

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