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Impact of dimethyl fumarate treatment on immune tolerance during Multiple Sclerosis

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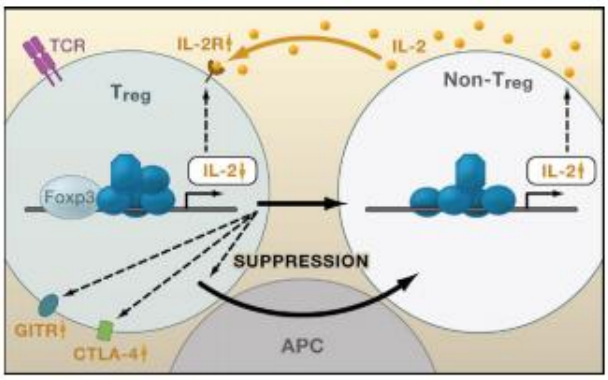
Abstract

Dimethyl fumarate (DMF) is a first line oral treatment for relapsing-remitting multiple sclerosis (RR-MS) patients. Although it is known that DMF have protective antioxidant properties against brain and spinal cord damage during MS, its precise mechanism of action remains still elusive. Here, we evaluated the immunological effects of DMF treatment in RR-MS patients and we investigated the impact of DMF on metabolic profile of T cells. We observed that DMF treatment results in reduction of number of total lymphocytes and of $CD19^+$ B cells, $CD4^+$ and $CD8^+$ T cells. In addition, DMF treatment is associated with a specific reduction of the memory T cells. Furthermore, we found that DMF treatment was able to increase the proliferative capacity of Treg cells over time and to restore the altered metabolic profile of T cells in RR-MS patients. Taken together, these data suggest a novel mechanism of action for DMF by modulation of the metabolic pathways of T cells that could lead to an improvement of immune tolerance during autoimmunity.

1. Introduction

1.1 Regulatory T cells and immune tolerance

The immune system uses many mechanisms to maintain immunologic *self*-tolerance and protect the host against responses to foreign antigens. Naturally occurring $CD4^+CD25^+FoxP3^+$ regulatory T (Treg) cells are critically involved in the maintenance of immune tolerance to *self*, immune homeostasis and are specialized in the control of immune and autoimmune response [1]. In 2003, Sakaguchi and colleagues identified the transcription factor FoxP3 (forkhead box P3), a member of the forkhead/winged-helix family of transcription factors, as a critical regulator of Treg development, function and homeostasis [2]. Their dysfunction (e.g. FoxP3 gene mutation) causes autoimmune and inflammatory diseases in humans and animals [3]. Treg cells constitutively express the high-affinity interleukin-2 (IL-2) receptor α chain CD25, and are able to suppress the activation, proliferation and effector functions, as also cytokine production, of several immune cells, including $CD4^+$ and $CD8^+$ T cells, B cells and antigen-presenting cells (APCs), natural killer (NK) cells, both *in vitro* and *in vivo* [4-5]. IL-2 has long been thought to be a major cytokine for T cell proliferation and differentiation based on its effects on T cell growth *in vitro* and it is vital and irreplaceable for the development, survival and function of $FoxP3^+$ natural Tregs. FoxP3, together with other transcription factors and coactivators/corepressors, represses the transcription of IL-2 in Tregs, rendering them highly dependent on exogenous IL-2 (mainly produced by activated non-Treg cells) for their maintenance and function. FoxP3 also activates the genes encoding Treg-associated molecules such as CD25, Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), and Glucocorticoid-Induced TNF-Receptor-related protein (GITR) and confers suppressive activity to Tregs, which directly suppress non Treg cells or modulate the function of APC to activate non-Treg cells [1] **(Fig. 1)**.



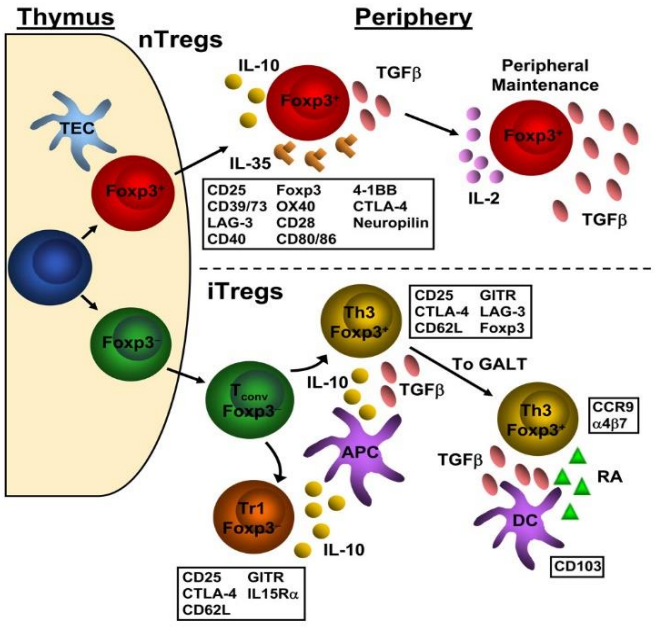
(Sakaguchi S. *et al.* Cell. 2008)

Fig. 1: Key roles of IL-2 in immune homeostasis. IL-2 produced by activated non-regulatory T cells contributes to the maintenance, expansion, and activation of natural Tregs, which in turn limits the expansion of non-regulatory T cells.

Although many studies indicate that CD25 is a crucial cell surface marker for the regulatory subset, other markers have been reported to be expressed on Treg cells essential for their function such as CD62 ligand (CD62L), CC-chemokine receptor 7 (CCR7) involved in lymph-node homing of naïve and Treg cells and programmed cell death 1 (PD-1), that is able to suppress T cell proliferation and induce peripheral tolerance. However, these markers have proven problematic for uniquely defining this specialized T cell subset in humans. Thus, several groups demonstrated that the IL-7 receptor (CD127) is down-regulated on a subgroup subset of $CD4^+$ T cells in peripheral blood and is inversely correlated with FoxP3 expression and suppressive capacity of Tregs. Therefore, a combination of CD4, CD25, and CD127 results in a highly purified population of T reg cells, which are highly suppressive in functional suppressor assays [6-7]. The importance of Tregs first became apparent in patients with mutations in FoxP3 who develop a severe, fatal systemic autoimmune disorder called Immune dysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome [8]. In addition to rare mendelian genetic diseases such as IPEX, it has become increasingly clear that Tregs are of tremendous importance to the pathogenesis of common human diseases. Defects in the *in vitro* suppressive function of Tregs occur in patients with numerous autoimmune diseases, including relapsing-remitting multiple sclerosis (RR-MS), type 1 diabetes (T1D), psoriasis, myasthenia gravis, and rheumatoid arthritis (RA) [9].

On the other hand, FoxP3^{+} Treg cells can also promote tumor progression by suppressing effective antitumor immunity [10]. Treg cell populations can be divided into two major groups according to their developmental origin: the thymus-derived Treg cells, known as nTreg cells, and those that are extrathymically derived, known as iTreg cells. Both nTregs and iTregs must achieve a fine balance between maintaining peripheral tolerance by suppressing potential autoimmune responses and also controlling responses to infections. The majority of FoxP3^{+} nTreg cells are produced by the thymus as an antigen-primed and functionally mature T cell subpopulation, specialized for immune suppression and comprise a small population ∼5–10% of peripheral CD4^{+}T cells [11].

nTregs differentiate from CD4^{+}CD25^{-} naïve conventional T (Tconv) cells to FoxP3^{+} Tregs in the thymus. In the periphery, natural Tregs express a number of cell surface markers. However, none of these cell surface markers are unique to Tregs as they are also found on activated conventional T cells. Natural Tregs utilize the cytokines IL-10, IL-35 and TGFβ to exert their suppressive effects upon conventional T cells. TGFβ and IL-2 have also been shown to be important to the maintenance and fidelity of the Treg signature. While iTregs can be generated from conventional T cell precursors. Once in the periphery, naïve conventional T cells can be induced to become FoxP3^{-}Tr1 cells or FoxP3^{+}Th3 cells via IL-10 and/or TGFβ secreted by APCs such as dendritic cells and macrophages. These induced Tregs share similar cell surface markers as natural Tregs. FoxP3^{+} induced Tregs can accumulate in the gut through upregulation of CCR9 and α4β7 via TGFβ and retinoic acid produced by CD103^{+} dendritic cells [12] **(Fig. 2)**.

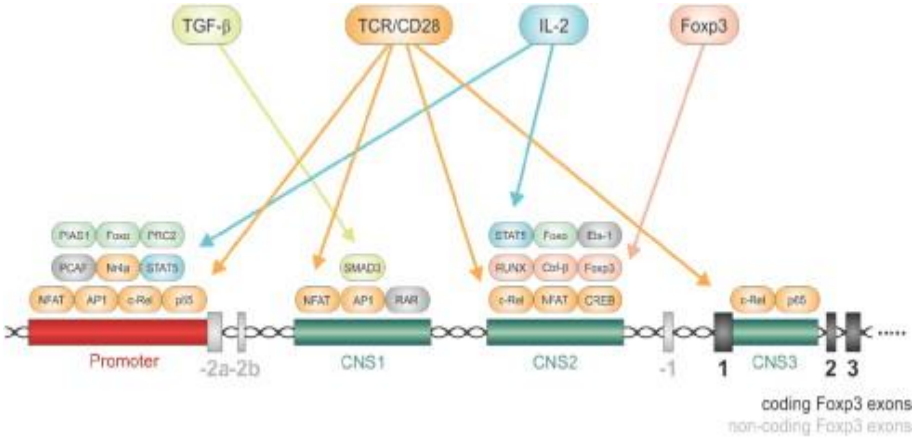


(Workman CJ. *et al.* Cell Mol Life Sci. 2009)

Fig. 2: Treg cells can either differentiate from naïve conventional T cells to FoxP3$^+$ Tregs in the thymus giving rise to thymus-derived Treg cells (nTreg) or in the periphery from naïve conventional FoxP3$^-$T cells giving rise to peripherally-induced Treg cells (iTreg).

1.2 FoxP3 in the control of the regulatory T cell lineage

FoxP3 is a master regulator of Treg development and suppressive function. The human full-length FOXP3 gene is located at the small arm of the X-chromosome (Xp11.23), with 11 different coding exons, 3 noncoding exons, and 104 introns [13]. After TCR stimulation, different Transcription Factors (TFs) (e.g. NFAT, AP1, CREB, Runx and c-Rel) are able to bind specific non-coding sequences (CNS1, CNS2 and CNS3), and determine FoxP3 gene regulation. Specifically, CNS1 contains binding sites for NFA-T and activator protein-1 (AP-1), CNS2 for the Runx1-core binding factor beta (CBF-beta) complex; their recruitment is necessary for FoxP3 expression in iTreg cells. TCR stimulation also leads to the binding of c-Rel to CNS3, which is required to open the FoxP3 promoter/enhancer region and promote its expression **(Fig. 3)**.



(Huehn J. *et al.* Semin Immunol. 2015)

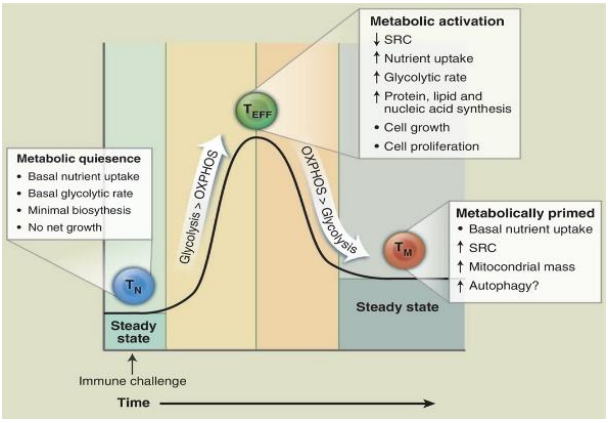
Fig. 3: Schematic diagram of transcriptional regulation of the FoxP3 locus. Regulatory regions of the FoxP3 locus including the promoter CNS1, CNS2, CNS3 and Transcription factors (TFs) that binding to each regulatory region.

DNA methylation and histone modification are two major epigenetic mechanisms that cooperate to modify chromatin structure. In particular, a conserved CpG island named Treg-specific demethylated region (TSDR) in the CNS2 region has also been found. The methylation status of this region regulates FoxP3 expression level and the stability of Treg cells. TSDR can lead to chromatin condensation when methylated, reducing the accessibility of DNA sequences and inhibiting the transcription of FoxP3. TSDR demethylation is essential for the transcription and the stability of FoxP3 in Treg cells, while partial methylation at the TSDR underlies the more labile FoxP3 expression seen in TGF-β-induced Treg [14-15]. The regulation of FoxP3 transcription is also related to the modification of the histone proteins. Histone acetylation is required for DNA transcription and its methylation inhibits DNA transcription [16]. FoxP3 can be also regulated at the protein level by ubiquitination, phosphorylation and other post-translational modifications, that are important in determining the structural and physical properties of this protein and the complex that it forms in Treg cells, with significant functional consequences on its expression [17-18]. In addition, through dimeric or inter-protein interactions, FoxP3 can form large protein complexes to regulate the expression of several genes associated with the Treg cell phenotype [19]. The presence of multiple FoxP3 splicing variants in humans represents an additional level of complexity in the biology of human FoxP3. To date, about 12 different FoxP3 transcripts have been described in humans, as well as 4 to 8 different splicing form the protein (ranging from 18 to 49 kDa).

However, the molecular events driving their expression, as well as the functional relevance of these proteins, has been not completely understood. The four main alternatively spliced isoforms of FoxP3 identified in human Treg cells are full-length (FoxP3FL), isoforms lacking exon 2 (FoxP3Δ2) or exon 7 (FoxP3Δ7) and isoforms lacking both exon 2 and exon 7 (FoxP3Δ2Δ7) [20-21].

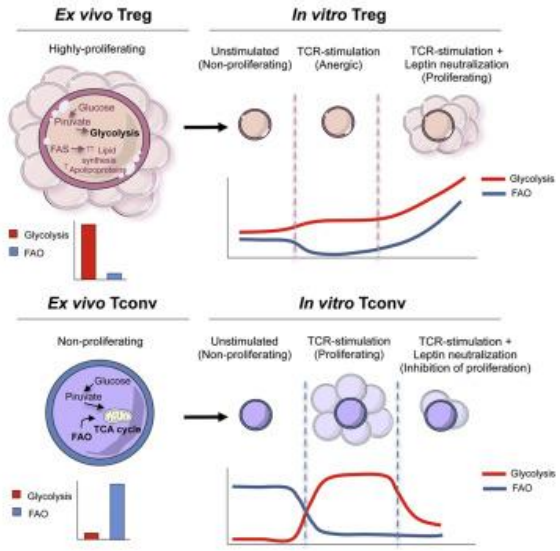
1.3 Metabolic control of T cell function

Compelling evidence indicate that the generation and function of T cells depend on underlying metabolic program [22-23]. In particular, resting naïve T cells, T cells that have not yet encountered antigen, and memory T cells maintain low rates of glycolysis and predominantly oxidize glucose-derived pyruvate via OXPHOS or engage fatty acid oxidation (FAO) to make ATP. Conversely, activation of T cells drives transcriptional changes, causing downregulation of oxidative metabolism and upregulation of biosynthetic pathways, such as aerobic glycolysis that promotes an increase in biochemical intermediates, necessary for nucleotide, amino acid, and fatty acid synthesis [24]. In this state, T cells are considered to be metabolically activated **(Fig. 4)** TCR signalling controls the metabolic reprogramming of naïve T cells through the up-regulation of glucose and amino acid transporters, facilitating nutrient uptake and T cell generation [25-26]. Treg and Tconv cells have a high degree of plasticity that associates with a different regulation of their own transcriptional program. In particular, *ex vivo* Treg cells were highly glycolytic rate associated with hyperactivation of the environmental sensor mammalian target of rapamycin (mTOR), while Tconv cells used predominantly fatty-acid oxidation (FAO). When cultured *in vitro*, Treg cells engaged both glycolysis and FAO to proliferate, while Tconv cell proliferation mainly relied on glucose metabolism [27] **(Fig. 5)**. Recent studies have shown that different metabolic programs are able to modulate FoxP3 expression, consequently, Treg lineage differentiation *in vivo* and *in vitro.* In particular De Rosa and colleagues demonstrated that glycolysis controlled the expression of splicing variants of FoxP3 containing the exon2 (FoxP3-E2), which is necessary for the suppressive function of human inducible Treg cells (iTreg).
Thus, inhibition of glycolysis impaired the generation and function of iTreg cells and reduced the induction of FoxP3 and the expression of Treg cell markers [28].



(Pearce E. L. *et al.* Science 2013)

Fig. 4: T cell metabolism changes over the course of an immune response.



(Procaccini C. *et al.* Immunity 2016)

Fig. 5: The metabolic signature of human Treg and Tconv cells.

These findings suggest that metabolism represents a critical checkpoint for T cell activation and function. Thus, an aberrant metabolic environment could influence the development of inflammation and autoimmune disorder, such as Multiple Sclerosis, by fueling the differentiation and activation of pathogenic T cells.

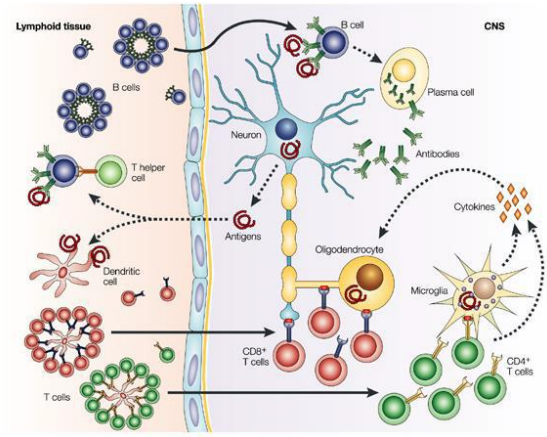
1.4 Multiple Sclerosis

Multiple Sclerosis (MS) is a common inflammatory disease of the central nervous system (CNS) characterized by myelin loss, gliosis, varying degrees of axonal pathology, and progressive neurological disfunction [29]. Clinical symptoms vary based on the site of neurologic lesions and often correlate with invasion of inflammatory cells across the blood-brain barrier with resulting demyelination and edema [30]. Though the pathogenesis is unclear, interplay between genetic, lifestyle and environmental factors play a major role [31]. The disease usually begins in young adulthood and the frequency is higher in women more than in men (2:1). Relapsing-remitting multiple sclerosis (RR-MS) is the most common initial presenting subtype, characterized by distinct relapses alternating with a period of either full recovery or incomplete recovery [32]. A variable proportion of patients with relapsing remitting MS (25–40%) develop secondary progressive disease over time with progressive accumulation of disability with infrequent or no relapses [33]. Primary progressive multiple sclerosis (PP-MS) (seen in approximately 10–15% patients) is defined by progressive accumulation of disability from the onset with no or minor relapses and typically presents with a progressive myelopathy with an older age of onset and involving a higher proportion of men [34]. Imaging studies have revealed differences between RR-MS and PP-MS. In patients that suffer from RR-MS, acute CNS lesions with spontaneous resolution are frequently detected, even in the absence of clinical attacks [35]**.** These lesions are usually located in areas of white matter and are often characterized by a disturbance of the blood–brain-barrier, local edema and demyelination- features that are compatible with an inflammatory process. By contrast, when progressing to the secondary phase and in patients with PP-MS, such inflammatory activity is much less evident [36].

These findings indicate that early in the disease, ongoing inflammatory activity is present in most patients and is responsible for the relapsing-remitting course, whereas a distinct process might be operative in the progressive phase of the disease, when inflammatory activity diminishes despite faster evolution of disability. Although MS is not an inherited disease, there is a strong genetic component to its etiology as evidenced by clustering of MS cases within families.

The risk of MS among first-degree relatives of MS patients is 10–50 times higher than the general population (absolute risk 2–5%); the concordance rate in monozygotic twins is about one-third [37-38]. The results of genomic screens in MS indicate that a considerable number of different genes, each having a relatively small contribution, are involved in the susceptibility to MS [39-40]. So far, only the human leukocyte antigen (HLA) class II alleles DR15/DQw6 (HLA-DRB1*1501; HLA-DQB1*0601), which code for molecules that participate in antigen recognition by T lymphocytes, have been consistently associated with MS in Caucasians [41]. More recently, alleles of interleukin-2 receptor alpha gene (IL2RA) and interleukin-7 receptor alpha gene (IL7RA) have also been identified as inheritable risk factors [42]. Furthermore, genetic susceptibility does not fully explain the changes in MS risk that occur with migration suggesting a likely role for environmental factors. In particular, environmental factors such as Epstein-Barr virus (EBV) infection and vitamin D deficiency have been extensively studied and strongly linked to MS risk. MS is prevalent in geographic areas farther away from the equator [43] and low vitamin D levels from reduced sun exposure may be a factor contributing to increased susceptibility to MS in these regions [44-45]. In fact, several studies have suggested that higher levels of vitamin D have a possible protective role in certain susceptible patient populations [46-47]. Moreover, it has been demonstrated that the risk of developing MS is approximately 15-fold higher among individuals with a history of EBV infection in childhood and about 30-fold higher among those infected with EBV in adolescence or later in life [48]. Hence, a combination of infectious agents and environmental and genetic factors has been postulated to be involved in disease pathogenesis. Several studies suggest that MS is an autoimmune disease [49]. The pathogenesis of MS involves immune attack against CNS antigens mediated through activated $CD4^+$ myelin-reactive T cells with a possible contribution by B cells. Much of our understanding of immunopathogenesis of MS is derived from the study of experimental autoimmune encephalomyelitis (EAE), an animal model of CNS inflammatory demyelination that can be induced by peripheral immunization with myelin protein components. EAE shares many of the histologic features of MS including active demyelination, oligodendrocyte and axonal loss, all of which are presumably mediated by myelin specific T cells [50].

The immunopathogenesis of MS is thought to involve a breach of *self*-tolerance toward myelin and other CNS antigens resulting in persistent peripheral activation of autoreactive T cells [30,51]. In particular, T- and B-cell responses are primed in the peripheral lymphoid tissue by antigens that are released from the CNS or by cross-reactive foreign antigens. Dendritic cells that present neural antigens are strong stimulators of T-cell responses. Clonally expanded B cells recognize their specific antigens, mature to plasma cells and release of immunoglobulin-γ (IgG) antibodies. These antibodies bind soluble antigen on expressing cells. While clonally expanded $CD8^+$ T cells also invade the brain and could encounter their specific peptide ligand, presented by glial or neuronal cells on MHC class I molecules. $CD4^+$ T cells migrate into the CNS and encounter antigens that are presented by microglial cells on MHC class II molecules. Reactivation of these cells leads to heightened production of inflammatory cytokines. These cytokines attract other immune cells, such as macrophages, which contribute to inflammation through the release of injurious immune mediators and direct phagocytic attack on the myelin sheath [49] **(Fig. 6)**.



(Hemmer B. *et al.* Nature Reviews Neuroscience 2002)

Fig. 6: Summary graph of the immunopathogenesis of MS.

One of the immune abnormalities observed in multiple sclerosis is a reduction in the suppressive function or number of Treg cells [52]. It has been demonstrated that Treg cell anergy (a state of hyporesponsiveness to antigenic stimulation) [53-54].

The anergic state of Treg cells *in vitro* partly depends on their high metabolic rate, which is associated with the activation of the mTOR pathway [23,55]. In particular, Procaccini and colleagues demonstrated that this nutrient-sensing metabolic pathway can be influenced directly by leptin [23]. In humans, leptin negatively controls the proliferation of Treg cells. Freshly isolated Treg cells produce consistent amounts of leptin and express high levels of the long signaling form of the leptin receptor, which constrains their proliferation. Neutralization of leptin *in vitro* reverses Treg cell hyporesponsiveness and triggers their proliferation and secretion of IL-2 upon TCR stimulation while at the same time inhibiting Tconv cell proliferation and IL-2 production [55]. The reduced *ex vivo* proliferation of RR-MS Treg cells is associated with altered interleukin-2 (IL-2) – IL-2 receptor (IL-2R) – signal transducer and activator of transcription 5 (STAT5) signaling and reduced expression of the 44 - and 47 - kDa forms of FoxP3, in conjunction with activation of the leptin-mTOR metabolic pathway, which has been linked to the hyporesponsiveness of Treg cells *in vitro* and *in vivo* [23,55]. Moreover, the impaired capacity of Treg cells to proliferate in RR-MS correlates with the clinical state of the subject, where increasing disease severity is associated with a decline in Treg cell expansion [56].

1.5 Dimethyl fumarate for the treatment of Multiple Sclerosis

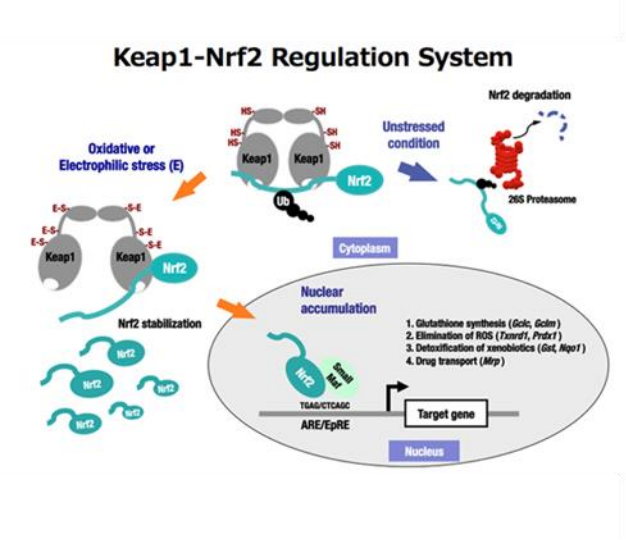
Seventeen disease-modifying therapies (DMTs) are currently available for treatment of RR-MS [57]. These include oral, self-injectable and infusible medications with varied mechanisms of action and degrees of efficacy [58]. Dimethyl fumarate (DMF; Tecfidera®) is an oral disease modulating treatment and the most prescribed drug for RR-MS in the U.S. In fact, two recently completed clinical trials, namely DEFINE and CONFIRM [59-60] have shown a strong efficacy of the drug by reducing relapse rates by about 50% as compared with placebo, and a significant reduction of gadolinium-enhanced lesions as well as T2 lesions on cranial MRI. The exact mechanism of action of DMF has not yet been fully elucidated.

Recent basic and clinical reports have shown that DMF inhibits immune cells and inflammation, and may have anti-oxidant properties protective against damage to the brain and spinal cord during MS.

Indeed, DMF acts on the nuclear erythroid-2-related factor (Nrf2) [61] and of nicotinic acid HCA2/GPRl09A receptors [62], which mediate these anti-inflammatory (immunomodulating) and neuroprotective effects. Inflammation and neurodegenerative processes are prominent early in the course of MS, so agents with putative dual anti-inflammatory and neuroprotective effects, such as delayed-release DMF, may be particularly useful.

1.6 Dimethyl fumarate antioxidative effects

Nrf2 is essential to maintain cellular redox homeostasis by regulating a number of genes involved in antioxidant protection such as glutathione [63], thioredoxin [64], heme oxygenase (HO1), and NAD(P)H dehydrogenase (NQO1) [65]. Under basal conditions, Nrf2 is mainly sequestrated in the cytoplasm by the cytoskeleton-associated protein Kelch-like ECH-associated protein 1 (Keap1). Keap1 forms an E3 ubiquitin ligase complex with Cullin 3 and Ring-box 1 and triggers polyubiquitination of Nrf2 by promoting its binding to Cullin 3 [66]. Ubiquitinated Nrf2 is then degraded by the 26S proteasome. Consequently, levels of Nrf2 protein are generally very low in basal-state cells. Under oxidative stress, monomethyl fumarate (MMF), which is an active metabolite of DMF, causes modification of cysteine residue 151 of Keap1.
Such oxidations probably cause conformational changes in the keap1-E3 ubiquitin ligase complex that blocks Nrf2 ubiquitination and favor its accumulation [67].
The modification of Keap1 then drives the dissociation and translocation of Nrf2 into the nucleus, initiating the transcription of many phase II antioxidant enzymes that contain the antioxidant response element (ARE) promoter sequence [68-69] (**Fig.7)**.
DMF has also been shown to prevent oxidative stress-induced apoptosis and promote survival of neural stem/progenitor cells and differentiated neurons by inducing Nrf2 ERK1/2-MAPK pathway [70].



(Taguchi K. *et al* Genes Cells. 2011)

Fig. 7: Molecular mechanisms of the Keap1–Nrf2 pathway in stress response.

1.7 Dimethyl fumarate immunomodulatory effects

Beyond targeting the Nrf2 pathway, DMF and, even more so, MMF were described as agonists of the hydroxycarboxylic acid receptor 2 (HCAR2), a G protein-coupled membrane receptor [71]. Activation of hydroxycarboxylic acid receptor 2 (HCAR2) by MMF in experimental autoimmune encephalomyelitis (EAE) mice led to a shift in molecular and functional phenotypes of activated microglia from pro-inflammatory to neuroprotective type. Hence, HCAR2-mediated activation of downstream AMPK–Sirt1 axis led to inhibition of NF-κB and of pro-inflammatory cytokine production. DMF administration to mice with EAE results in upregulation of microglia markers associated with neuroprotection and mitigation of alterations in synaptic transmission [72]. In addition, DMF exhibits immunomodulation by biasing toward $CCR3^{+}$Th2 subsets of T cells. This leads to an increase in anti-inflammatory cytokines such as IL-4 and a decrease in pro-inflammatory cytokines IFN-γ and IL-17 [73]. It has also been reported that DMF alter regulatory T cell population by specifically enhancing peripheral regulatory T (pTreg) cell subset, which is known to contribute to peripheral tolerance [74].

2. Aim of the study

In this study, we will evaluate the effect of DMF treatment on peripheral blood immune phenotype, survival/migration of T cells and on Treg cells proliferative capacity in RR-MS patients. Since the active form of DMF is fumarate, a TCA cycle metabolite, this treatment may act also by altering T cell metabolism. Therefore, we will also investigate the effect of DMF treatment on intracellular glycolysis and mitochondrial respiration in T cells from the above patients.

Understanding the precise mechanism of action of DMF raises the possibility to improve DMF potential and its therapeutic implication not only in MS but also in other autoimmune disorders.

3. Materials and methods

3.1 Patients and study design

The study was approved by the Institutional Review Board of the Università degli Studi di Napoli "Federico II". RR-MS patients were enrolled by our clinician collaborators at Università degli Studi di Napoli "Federico II", AOU "Antonio Cardarelli" Napoli and IRCCS Istituto Neurologico Mediterraneo (INM) Neuromed, Pozzilli (IS). Peripheral blood was obtained from 9 naïve-to-treatment RR-MS patients (patients naïve to any pharmacological treatment, either corticosteroid treatment or other drugs) and from 35 RR-MS patients previously treated with other drugs. All the patients started DMF treatment after the enrollment and they signed a written informed consent approved by Institutional Review Boards after the enrollment. Blood samples were collected at 9:00 a.m. into heparinized Vacutainers (BD Biosciences) and were processed within the following 4 h. All patients were defined with RR-MS diagnosis, according to the revised McDonald's criteria [33].

Inclusion criteria

-Patients with RR-MS, which have had at least one relapse in the last year of illness.

-Treatment naïve or free from first line disease-modifying therapies for at least one month.

-EDSS up to 5.5.

-Age between 18 and 55 years.

-Adequate liver function (ALT: 7-55 U/L for males and 7-45 U/L for females/ AST: 8-48 U/L for males and 8-43 U/L for females).

Exclusion criteria

-Medical and psychiatric conditions that impair the patients' ability to provide informed consent.

-Concomitant serious medical illnesses, especially severe liver disorders, anemia, immuno-deficiency and infections.

-History of cancer (except for basal or squamous cell skin lesions which have been surgically excised, with no evidence of metastasis).

-Pregnancy and lactation.

At enrollment demographic (age and gender) and disease information (disease duration, previous treatments, comorbidities and concomitant therapies, EDSS score, number of relapses and an MRI scan) were obtained. Blood samples were obtained at baseline and after 9, 18 and 24 months starting DMF therapy, together with information about relapses and EDSS score.

3.2 Immunophenotypic analysis

Immunophenotypic analysis of peripheral blood of all RR-MS patients before and after 9, 18, 24 months treatment with DMF was performed with an EPICSXL flow cytometer (Beckman Coulter) using the Beckman Coulter software program XL system II. Triple combinations of different anti-human mAbs, e.g., FITC- and phycoerythrin(PE)-anti-CD3, PE- and PC5-anti-CD4, PC5-anti-CD8, PE-anti-CD16, PC5-anti-CD19, PE-anti-CD25, FITC-anti-CD45, and PE-anti-CD56 (all from Coulter Immunotech, Marseille, France), were used for immunofluorescence staining and to identify different cell populations.

3.3 Multiplex-based immunoassay for plasma cytokine

All plasma samples from RR-MS patients before and after 9, 18 and 24 months DMF treatment were obtained via centrifugation and stored at −80°C before the analysis. Human Custom ProcartaPlex 13-plex (NOVEX, Life technologies) was used for the evaluation of the levels of Eotaxin, Interleukin [IL]-6, IL-8, IFN-γ-inducible Protein (IP)-10, Leptin, Monocyte Chemoattractant Protein (MCP)-1, Monokine Induced by IFN-γ (MIG), Macrophage Inflammatory Proteins (MIP)-1alpha, (MIP)-1beta, Plasminogen Activator Inhibitor-1 (PAI-1), Resistin, Growth-related alpha protein (Gro-alpha/KC) and Ghrelin. Fluorescence was detected using Luminex 200 System (Luminex, Austin, TX), and results were analyzed using the xPONENT Software Version 3.1 (Luminex).

3.4 Evaluation of cell death

Freshly isolated peripheral blood mononuclear cells (PBMCs) from RR-MS patients, before and after treatment with DMF, were surface-stained with the following mAbs: FITC Annexin V, PerCP-Cy 5.5 PI (Propidium Iodide), Pe-Cy-7 mouse anti-human CD45RA (clone Hl100), V500 mouse anti-human CD4 (clone RPA-T4) and BV421 mouse anti-human CD8 (clone RPA-T8) all from BD Biosciences. Subsequently cells were washed with Annexin V binding buffer (BD Biosciences). Analyses were performed with Diva software (BD) and the Flow-Jo Software (Tree Star Inc.).

3.5 Flow cytometry analysis of proliferation, metabolism and migration markers

Freshly isolated PBMCs from RR-MS patients, before and after treatment with DMF, were surface-stained with the following mAbs: APC-H7-conjugated anti-human CD4 (clone RPA-T4), BB515 rat anti-human CXCR5 (CD185) (clone RF8B2), Pe-Cy-7 mouse anti-human CCR4 (CD194) (clone 1G1), BV421 mouse anti-human CCR8 (CD198) (clone 433H), PerCP-Cy 5.5 mouse anti-human CCR10 (clone 1B5), BV421 mouse anti-human CCR7 (CD195) (clone 150503), Alexa Fluor 647 mouse anti-human CCR9 (CD199) (clone 112509) and BV510 mouse anti-human CD62L (clone DREG-56) all from BD Biosciences. Subsequently cells were washed, fixed, permeabilized (fixation-permeabilization buffer BD Biosciences) and stained with PE mouse anti-human FoxP3 (clone 259D/C7), BV510 mouse anti-Ki67 (clone B56) both from BD Biosciences and Alexa Fluor 647 phospho-S6 ribosomal protein Ser235/236 (clone D57.2.2E from Cells Signaling) monoclonal antibodies. Analyses were performed with the Diva software (BD) and the Flow-Jo Software (Tree Star Inc.).

3.6 Cell purification, cultures and proliferation assay

Peripheral blood mononuclear cells (PBMCs) from all RR-MS patients of either groups, before and after treatment with DMF, were isolated after Ficoll–Hypaque gradient centrifugation (GE Healthcare).

Human Treg ($CD4^+CD25^+CD127^-$) and Tconv ($CD4^+ CD25^-$) cells were purified from PBMCs by magnetic cell separation with a regulatory $CD4^+CD25^+$ T Cell Kit (Thermo Fisher Scientific).

For proliferation assays, we cultured both Treg cells and Tconv (1×10^4 cells per well) in round-bottom 96-well plates (Becton Dickinson Falcon) with RPMI 1640 medium supplemented with 100 UI ml^{-1} penicillin and 100 ml^{-1} streptomycin (all from Life Technologies) and 5% patient autologous serum (AS). Cells were stimulated for 3 days in the presence of anti-CD3 and anti-CD28-coated Dynabeads (0.5 beads per cell; Thermo Fisher) in the presence or absence of anti-leptin neutralizing mAb (clone 44802, cat. #mAb 398 R&D systems) at a final concentration of 20 μg/ml. On the last day (48h), we added [^{3}H] thymidine (0.5 μCi per well) (Amersham-Pharmacia Biotech) to the cultures and harvested cells after 12h (Tomtec). Radioactivity was measured with a beta-plate scintillation counter (Wallac).

3.7 Seahorse metabolic analysis

The metabolic profile was evaluated in PBMCs from RR-MS patients, before and after treatment with DMF. PBMCs were plated in XF-96 plates (Seahorse Bioscience) at a density of 4×10^5 cells per well and cultured in RPMI 1640 medium supplemented with 5% AS for 12h in the presence of anti-CD3 (OKT3). Real-time measurements of extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were made with an XFe96 Extracellular Flux Analyzer (Seahorse Bioscience). ECAR was measured in XF medium in basal conditions and in response to glucose (10 mM), oligomycin (5 μM) and 2-deoxy-d-glucose (2-DG) (100 mM) (all from Sigma-Aldrich). OCR was measured in XF media (non-buffered DMEM medium, containing 10 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate), under basal conditions and in response to 5 μM oligomycin, 1.5 μM of carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) and 1 μM of Antimycin and Rotenone (all from Sigma Aldrich). Experiments with the Seahorse system were done with the following assay conditions: 3 min of mixture; 3 min of waiting; 3 min of measurement. Indices of glycolytic pathway activation were then calculated from ECAR profile: basal glycolysis post glucose injection (after glucose addition), maximal glycolysis (after oligomycin addition) and glycolytic capacity (calculated as the difference between oligomycin-induced ECAR and 2DG-induced ECAR).

Indices of mitochondrial respiratory function were calculated from OCR profile: maximal respiration OCR (calculated as the difference of FCCP rate and antimycin + rotenone rate), ATP-linked OCR (calculated as the difference between basal OCR rate and oligomycin-induced OCR rate) and spare capacity OCR (calculated as the difference of FCCP rate and basal OCR rate).

3.8 Statistical analysis

Statistical analysis will be performed using Prism GraphPad 6.0. Data will be tested for normality distribution through the D'Agostino and Pearson omnibus normality tests. Differences between patients before and after pharmacological treatment will be analysed using Mann–Whitney test or Wilcoxon matched-pairs signed rank test, as appropriate. The significance level is established at $p \leq 0.05$.

4. Results

4.1 Clinical and anthropometric characteristics of RR-MS patients

We included in the study 44 RR-MS patients (16 males and 28 females) according to current diagnostic criteria [33]. 9 patients were naïve to any pharmacological treatment and 35 were already treated with other drugs (6 patients with Avonex®, 12 patients with Rebif®, 11 patients with Tysabri® and 6 patients with Gilenya®).
After the enrollment in the study all the patients started DMF treatment, according to the clinical practice. Patient demographic and clinical characteristics are reported in **Table I**. At baseline visit, MS clinical features were recorded: disease duration (years since clinical onset), disease course, expanded disability status scale (EDSS) [75] and annualized relapse rate in the previous 2 years. We followed-up the population according to clinical practice 9, 18 and 24 months after the enrollment and upon DMF treatment, recording EDSS and clinical relapses. A relapse was defined as newly developing symptoms or reactivation of pre-existing deficits occurring at least 30 days after the preceding episode for a minimum of 24h, in the absence of increased body temperature or infections. Corticosteroid treatment was allowed for relapses according to clinical practice and was recorded.

Table I. Demographic, clinical characteristics and treatment of 44 RR-MS patients.

Male, n (%)	16 (36.4)
Female, n (%)	28 (63.6)
Age at onset, mean ± SD, year	47.5 ± 9.38
BMI, mean ± SD, kg/m^2	24.9 ± 4.5
Disease duration, mean ± SD, year	12.5 ± 90
Naïve-to-treatment, n (%)	9 (20.5)
Switched from other therapies, n (%):	35 (79.5)
- Avonex n (%) - Rebif n (%) - Tysabri n (%) - Gilenya n (%)	6 (17.1) 12 (34.3) 11 (31.5) 6 (17.1)
Naïve-to-treatment:	
EDSS at baseline, median [min; max]	2 [1.5; 3]
EDSS at 24 months of treatment with DMF, median [min; max]	1.5 [1; 2.5]
Switched from other therapies:	
EDSS at baseline, median [min; max	3.5 [1.5; 7]
EDSS at 24 months of treatment with DMF, median [min; max]	3.5 [1.5; 7]

BMI: body mass index; EDSS: expanded disability status scale.

4.2 Immunological profiling of RR-MS patients before and after DMF treatment

We performed immunophenotype of peripheral blood cells from all RR-MS patients by multiparametric cytofluorimetric analysis, to determine any possible changes in the number and percentage of different cell subsets before and after 9, 18 and 24 months of DMF treatment **(Fig. 8-9 and 10-11)**.

We observed that naïve-to-treatment RR-MS patients showed a significant decrease of the number and percentage of lymphocytes **(Fig. 8B and 9A)**, $CD3^+$ **(Fig. 8C and 9B)** and $CD8^+$ T cells **(Fig. 8E and 9D)** after DMF treatment overtime. Also, we observed that naïve-to-treatment RR-MS patients showed a reduction in the number of $CD4^+$, $CD19^+$ and $CD8^+CD11b^+$ suppressor T cells after DMF treatment **(Fig. 8D, 8G and 8O)**. Moreover, treatment with DMF was associated with a significant increase in the percentage of $CD16^+CD56^+$ (NK) cells **(Fig. 9E)**. After 9 and 18 months from the enrollment there was an increase in the percentage of $CD3^+CD16^+CD56^+$ T cells, a subset involved in the control of immune tolerance, induced by DMF **(Fig. 9M)**.

In addition, we observed a significant reduction in the absolute number and percentage of $CD3^+$ and $CD4^+$ T cells with a memory phenotype ($CD3^+CD45RO^+$ and $CD4^+CD45RO^+$ T cells) in naïve-to-treatment RR-MS patients after DMF treatment **(Fig. 8J-9I and 8L-9K)**. Concomitantly, we found that DMF treatment caused a significant increase in the percentage of $CD3^+$ and $CD4^+$ T cells with a naïve phenotype ($CD3^+CD45RA^+$ and $CD4^+CD45RA^+$ T cells) **(Fig. 9H-9J)**, despite a reduction of the number of $CD3^+CD45RA^+$ T cells has been observed **(Fig. 8I)**. Moreover, we assessed the effect of DMF on $CD8^+$ T cells with naïve $CD8^+CD45RA^+$ and memory phenotype $CD8^+CD45RO^+$. Of note, treatment with DMF was able to reduce the number of naïve $CD8^+CD45RA^+$ T cells **(Fig. 8Q)** and the number and percentage of memory $CD8^+CD45RO^+$ T cells **(Fig. 8R and 9Q)**.

We performed the same analyses also in RR-MS patients previously treated with other drugs and switched to DMF treatment after the enrolment in the study **(Fig. 10-11)**. In this group of patients, we observed a significant reduction in the number of leucocytes **(Fig. 10A)** after DMF treatment. In addition, DMF treatment was able to reduce the number and percentage of total lymphocytes, $CD3^+$ and $CD8^+$ T cells **(Fig. 10B-11A, 10C-11B and 10E-11D)** and the number of $CD4^+$,$CD4^+CD8^+$, $CD4^+CD25^+$, $CD3^+CD16^+CD56^+$, $CD8^+CD11b^+$ and $CD3^-CD8^+$ T cells **(Fig. 10D, 10H, 10M, 10N, 10O and 10P)**.

In addition, we found that treatment with DMF caused a statistically significant reduction in the number of $CD16^+CD56^+$ and $CD19^+$ cells **(Fig. 10F and 10G, respectively)**, although the percentage of such subsets was increased by DMF **(Fig. 11E and 11F, respectively)**. We next analyzed naïve and memory cells. The analysis revealed that DMF treatment caused a specific reduction in the number and percentage of $CD3^+$ and $CD4^+$ T cells with a memory phenotype ($CD3^+CD45RO^+$ and $CD4^+CD45RO^+$ T cells) **(Fig. 10J-11I and 10L-11K)**, and an increase in their naïve compartment ($CD3^+CD45RA^+$ and $CD4^+CD45RA^+$ T cells) **(Fig. 11H and 11J, respectively)**. Also, in this group of patients, we observed that DMF treatment was able to reduce the number of naïve phenotype ($CD3^+CD45RA^+$ T cells) **(Fig. 10I)**. Moreover, we found that DMF treatment induced a reduction in the number of naïve $CD8^+$ T cells ($CD8^+CD45RA^+$) **(Fig. 10Q)** together with a reduction in the number and percentage of memory $CD8^+$ T cells ($CD8^+CD45RO^+$) **(Fig. 10R and 11Q)**.

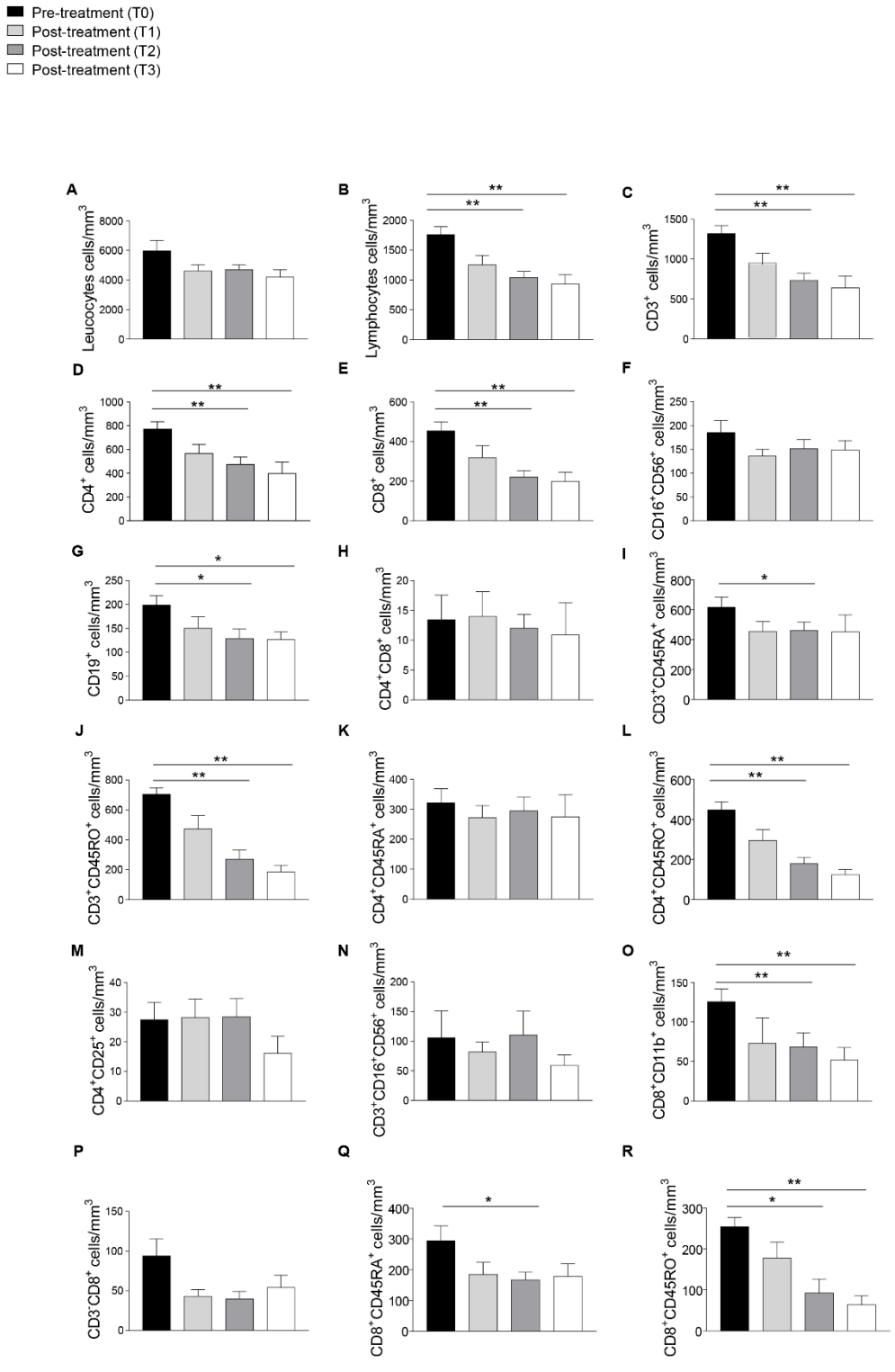


Fig. 8: Number (cells/mm^3) of peripheral blood immune cell populations in naïve-to-treatment RR-MS patients before (T0) and after 9, 18 and 24 months (T1, T2, T3) of DMF treatment. Data are shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$ by Wilcoxon matched-pairs signed rank test the effect of DMF treatment to the baseline.

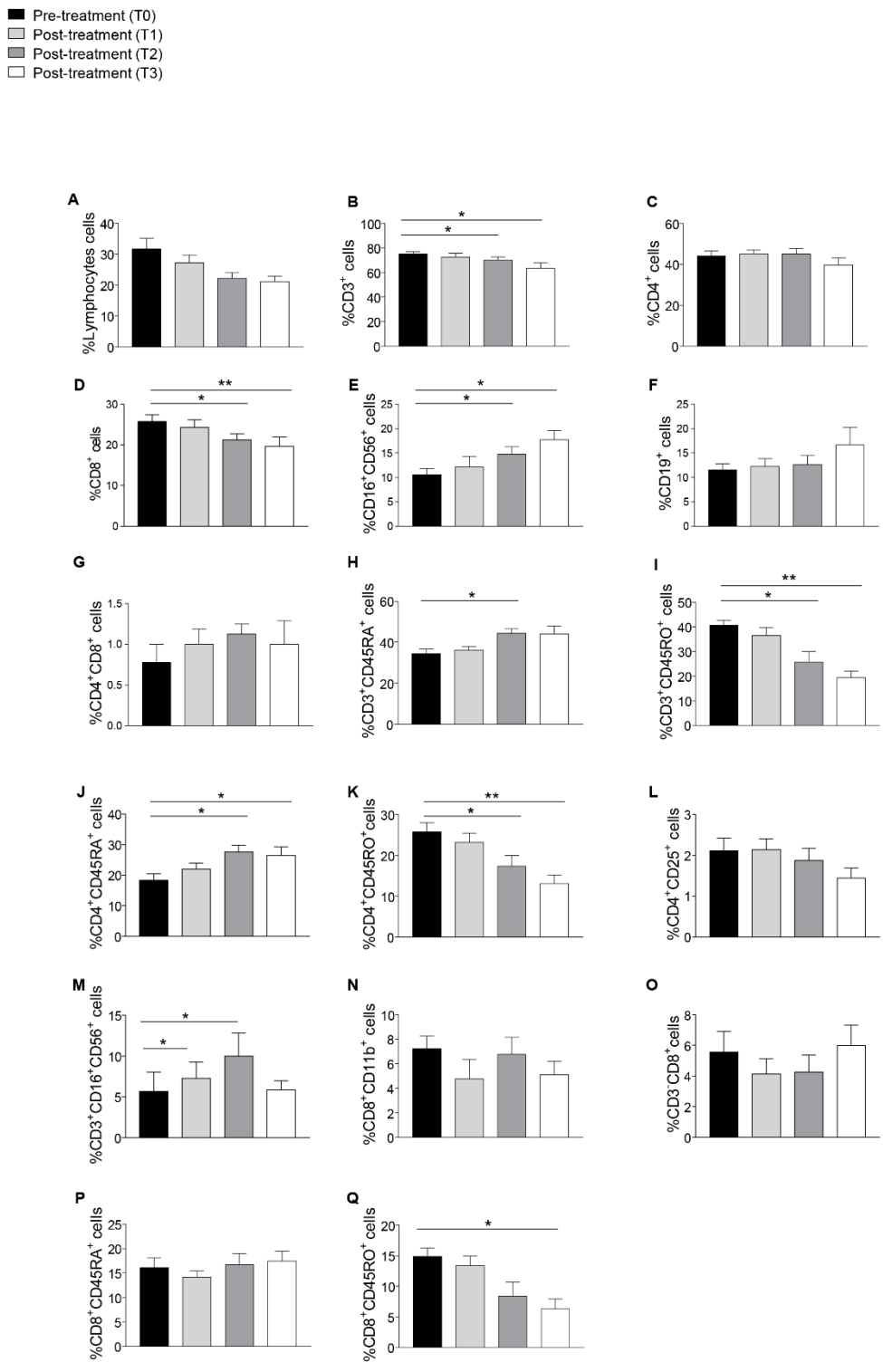


Fig. 9: Percentage on lymphocytes of peripheral blood immune cell populations in naïve-to-treatment RR-MS patients before (T0) and after 9, 18 and 24 months (T1, T2, T3) of DMF treatment. Data are shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$ by Wilcoxon matched-pairs signed rank test comparing the effect of DMF treatment to the baseline.

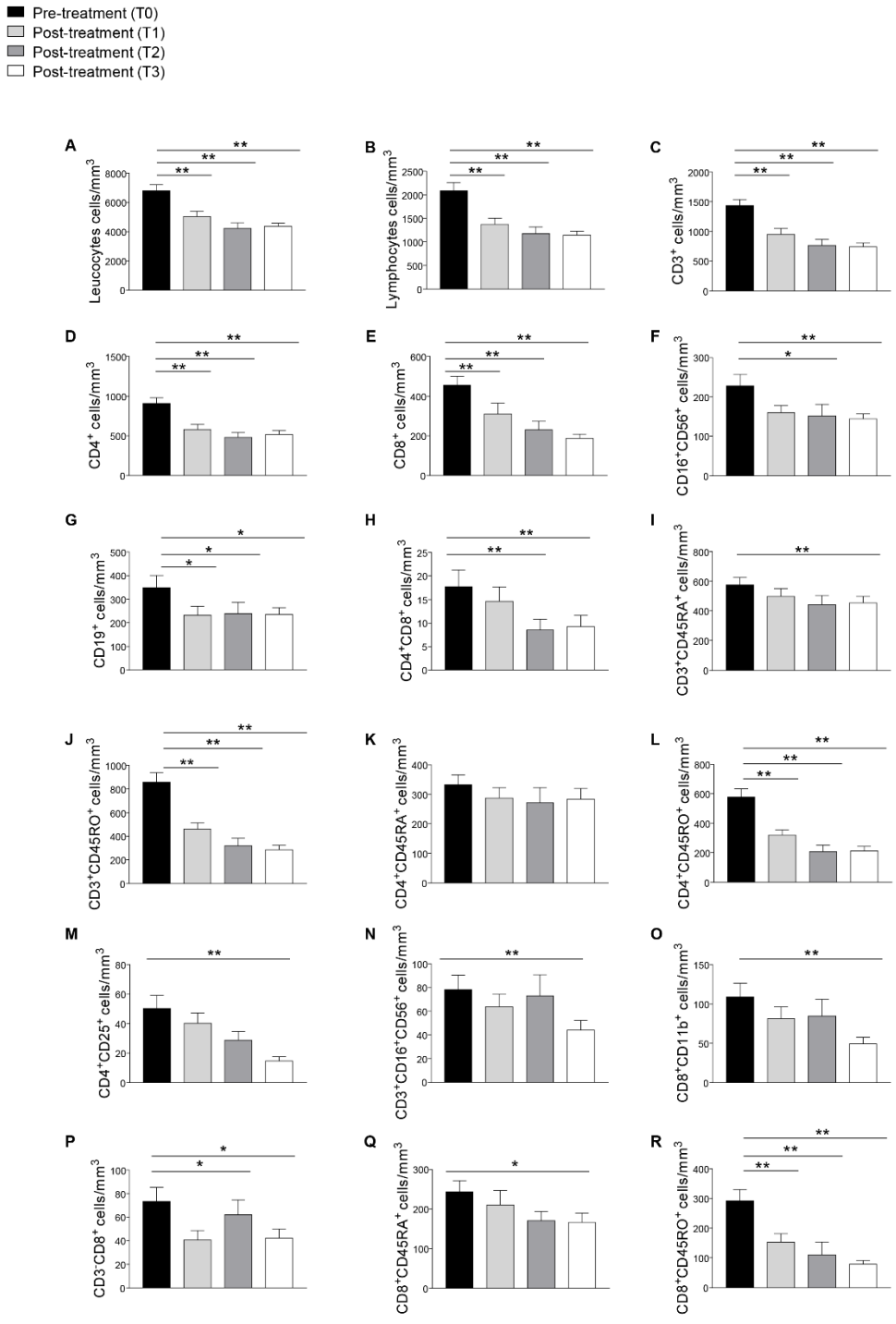


Fig. 10: Number (cells/mm^3) of peripheral blood immune cell populations in RR-MS patients, switched from previous treatment, before (T0) and after 9, 18 and 24 months (T1, T2, T3) of treatment with DMF. Data are shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$ by Wilcoxon matched-pairs signed rank test comparing the effect of DMF treatment to the baseline.

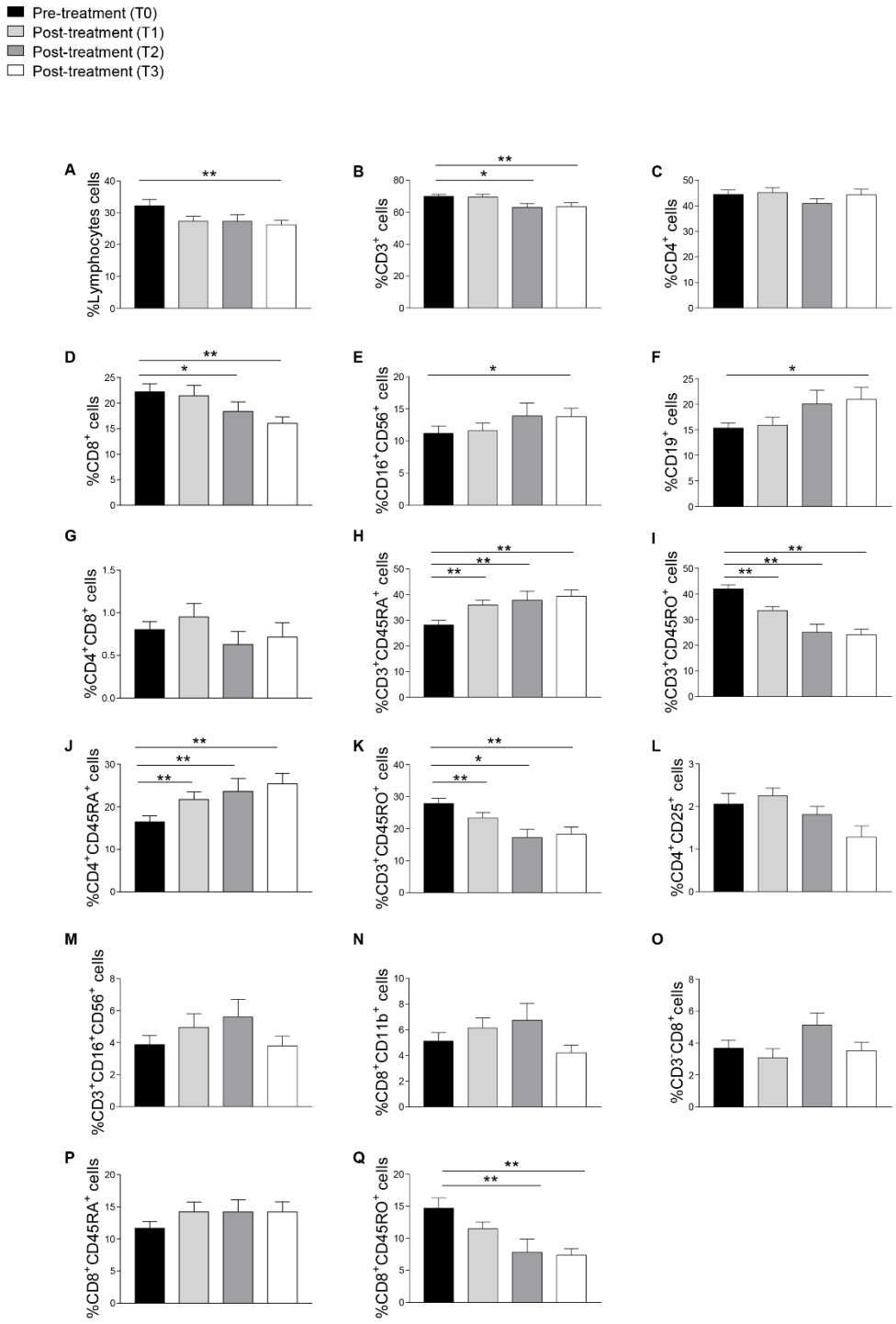


Fig. 11: Percentage on lymphocytes of peripheral blood immune cell populations in RR-MS patients, switched from previous treatment, before (T0) and after 9, 18 and 24 months (T1, T2, T3) of DMF treatment. Data are shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$ by Wilcoxon matched-pairs signed rank test comparing the effect of DMF treatment to the baseline.

4.3 Effect of DMF on plasma factors in RR-MS patients

To assess whether DMF treatment in RR-MS was associated with specific modulation of plasma cytokine/chemokine/adipocytokine levels, we evaluated the concentration of Eotaxin, Interleukin [IL]-6, IL-8, IFN-γ-Inducible Protein (IP)-10, Leptin, Monocyte Chemoattractant Protein (MCP)-1, Monokine Induced by IFN-γ (MIG), Macrophage Inflammatory Proteins (MIP)-1alpha, (MIP)-1beta, Plasminogen Activator Inhibitor-1 (PAI-1); Resistin, Growth-related oncogene (Gro-alpha/KC) and Ghrelin in RR-MS patients before and after DMF treatment. We evaluated the plasma factors profile of both naïve-to-treatment patients **(Fig. 12)** and of patients previously treated with other drugs **(Fig. 13)**. In naïve-to-treatment RR-MS patients, we observed a significant increase of PAI-1 **(Fig. 12J)** after 18 and 24 months of DMF treatment. In addition, we observed a trend toward a reduction of IL-6 **(Fig. 12B),** IL-8 **(Fig. 12C),** MIP-1alpha **(Fig. 12H)** and MIP-1beta **(Fig. 12I**) after 24 months of treatment with DMF. We performed the same analyses also in RR-MS patients previously treated with other drugs and switched to DMF treatment after the enrollment in the study.
We found that DMF treatment was associated with a significant reduction of IL-8 **(Fig. 13C)**, Resistin **(Fig. 13K)** and Ghrelin **(Fig. 13L)** after two years of treatment (T3).
In addition, we observed a reduction of MIP-1alpha **(Fig. 13H)** and an increase of MIG (**Fig. 13G)** after 9 months of treatment (T1). We also reported a trend toward a reduction of IL-6 (**Fig. 13B**), Leptin **(Fig. 13E)** and MIP-1beta **(Fig. 13I)** after DMF treatment.

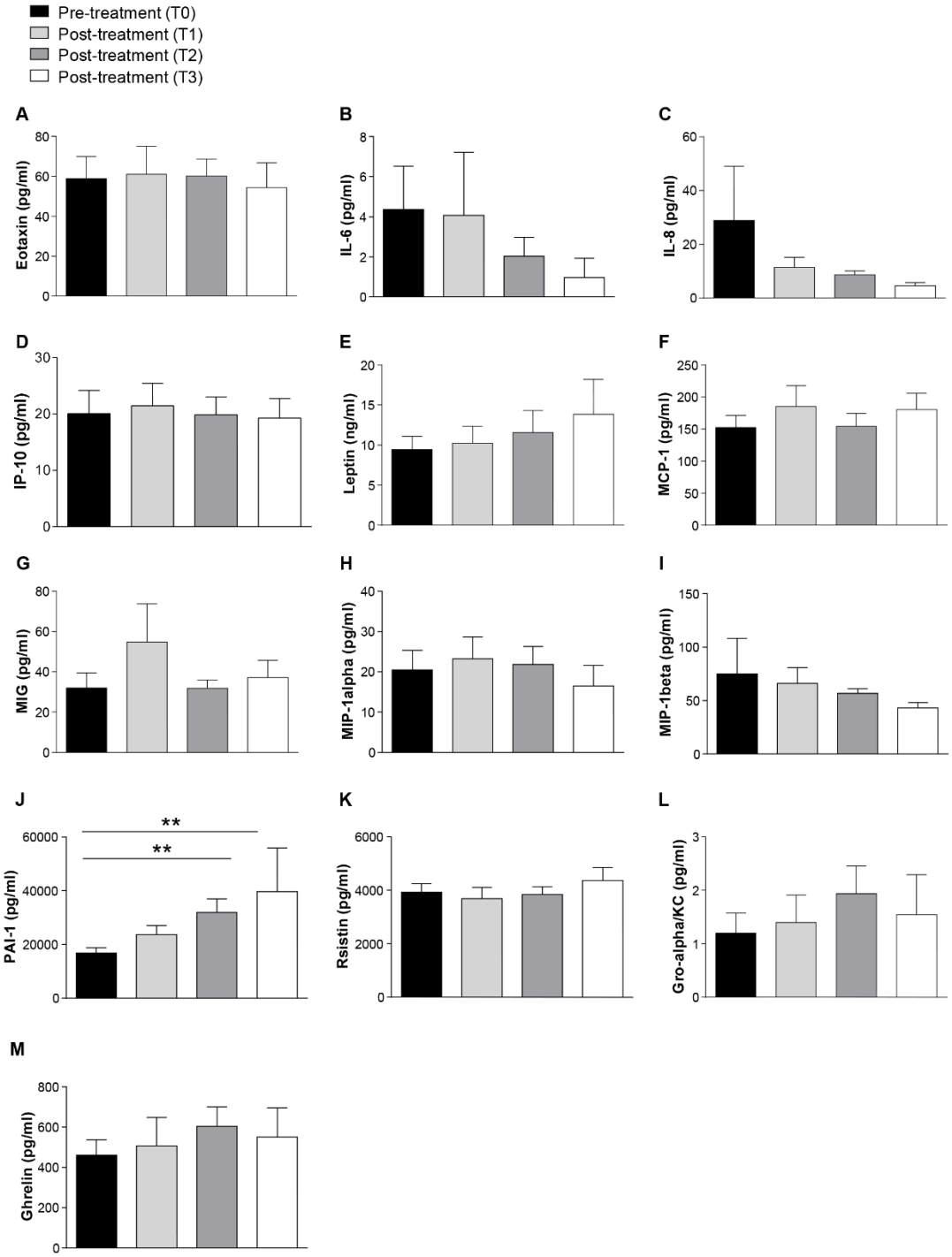


Fig. 12: Plasma cytokine/chemokine/adipocytokine levels in naïve-to-treatment RR-MS patients before (T0) and after 9, 18 and 24 months (T1, T2, T3) of DMF treatment. Data are shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$ by Wilcoxon matched-pairs signed rank test comparing the effect of DMF treatment to the baseline.

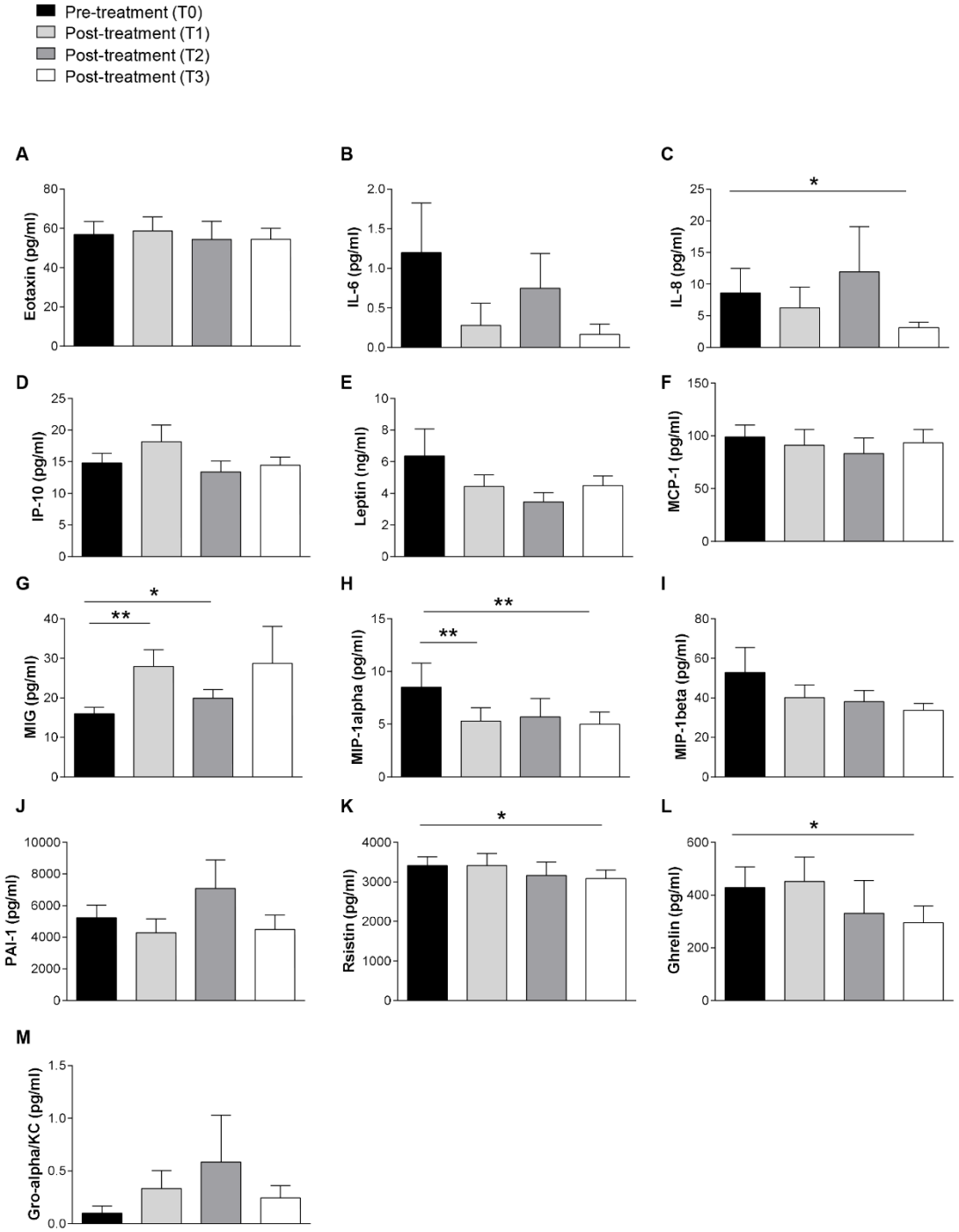


Fig. 13: Plasma cytokine/chemokine/adipocytokine levels in RR-MS patients, switched from previous treatment to DMF, before (T0) and after 9, 18 and 24 months (T1, T2, T3) of DMF treatment. Data are showed as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$ by Wilcoxon matched-pairs signed rank test comparing the effect of DMF treatment to the baseline.

4.4 DMF treatment was associated with increased apoptosis rate of $CD4^+$ and $CD8^+$ T cells

Since we observed that DMF treatment was associated with a modulation of the number and percentage of naïve and memory T cell subsets *in vivo*, we investigated whether the reduction of these cell subsets was associated with increased induction of cell death. For this purpose, PBMCs isolated from patients before and after 3 months of DMF treatment were analyzed for their positivity to Propidium Iodide (PI) and Annexin V. In order to evaluate the percentage of necrotic and apoptotic cells in the different cell compartments, we performed the analysis on both naïve and memory $CD4^+$ and $CD8^+$ T cells **(Fig. 14)**. DMF treatment induced a significant increase of Annexin V^+ $CD4^+$ and $CD8^+$ T cells with both a naïve ($CD4^+CD45RA^+$ and $CD8^+CD45RA^+$) **(Fig. 14B and 14F)** and memory ($CD4^+CD45RA^-$ and $CD8^+CD45RA^-$) phenotype **(Fig. 14D and 14H)**. Although the induction of Annexin V expression was observed in both naïve and memory $CD4^+$ T cells after DMF treatment, we reported a higher degree of apoptosis induction in memory compartment **(Fig. 14I**). We didn't observe this different trend in $CD8^+$ T cells **(Fig. 14J)**. In addition, RR-MS patients showed a statistically significant reduction of PI^+ $CD8^+$ T cells with a naïve phenotype ($CD8^+CD45RA^+$) after DMF treatment **(Fig. 14E)**.

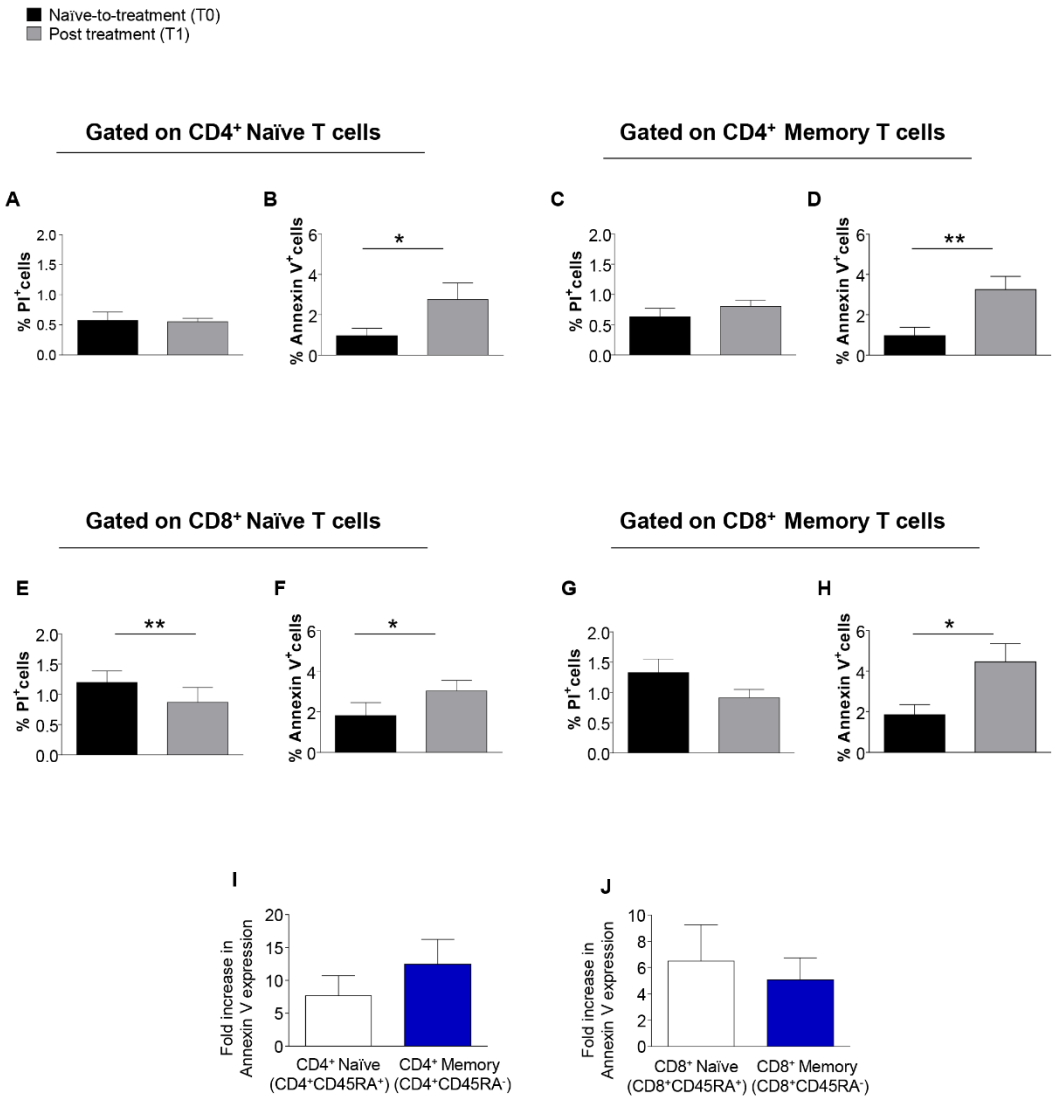


Fig. 14: Percentage of Propidium Iodide (PI) and Annexin V positive cells gated on $CD4^+CD45RA^+$ (A and B), $CD4^+CD45RA^-$ (C and D) and $CD8^+CD45RA^+$ (E and F), $CD8^+CD45RA^-$ (G and H) in freshly isolated PBMCs from naïve-to-treatment RR-MS patients before (T0) and after 3 months of DMF treatment (T1). Data are shown as mean ± SEM. $*p \leq 0.05$, $**p \leq 0.01$ by Mann – Whitney test. Fold increase in Annexin V expression after DMF treatment in naïve and memory $CD4^+$ cells (I) and in naïve and memory $CD8^+$cells (J).

4.5 DMF treatment induced a modulation of Ki67 and CD62L expression

To evaluate whether the modulation in the number of peripheral blood cell populations was associated with an increase of cell migration versus peripheral organs/ tissues after DMF treatment, we analyzed the expression of several chemokine involved in cell trafficking including CXCR5, CCR10, CCR7, CCR9, CD62L, CCR4 and CCR8 in different subsets of $CD4^+$ T cells ($CD4^+FoxP3^+$ with a regulatory phenotype and $CD4^+FoxP3^-$ cells defined as conventional T cells) from RR-MS patients, before and after 3 months of DMF treatment **(Fig. 15)**. The analysis revealed a significant increase of CD62L expression in both $CD4^+FoxP3^+$ and $CD4^+FoxP3^-$ cells after DMF treatment (**Fig. 16G and 16P)**. We didn't report any significant differences in the expression of the other migratory markers analyzed in both subsets **(Fig. 16)**. We next evaluated the expression of the proliferation marker Ki67 in both $CD4^+FoxP3^+$ and $CD4^+FoxP3^-$ cells. We observed that after 3 months of treatment with DMF there was a reduction in the proliferation of $CD4^+FoxP3^+$ and $CD4^+FoxP3^-$ cells, as indicated by a decreased expression of Ki67 **(Fig. 16A and 16J)**. Since the mammalian target of rapamycin (mTOR) kinase, is an intracellular metabolic sensor that controls several processes including proliferation, differentiation and cellular functions and migration, we next evaluated the effect of DMF treatment on S6 phosphorylation, a direct target of mTOR. We observed a trend towards a decrease of p-S6 expression in the $CD4^+FoxP3^+$ **(Fig. 16B)** and an increase of its expression in $CD4^+FoxP3^-$ cells **(Fig. 16K)**.

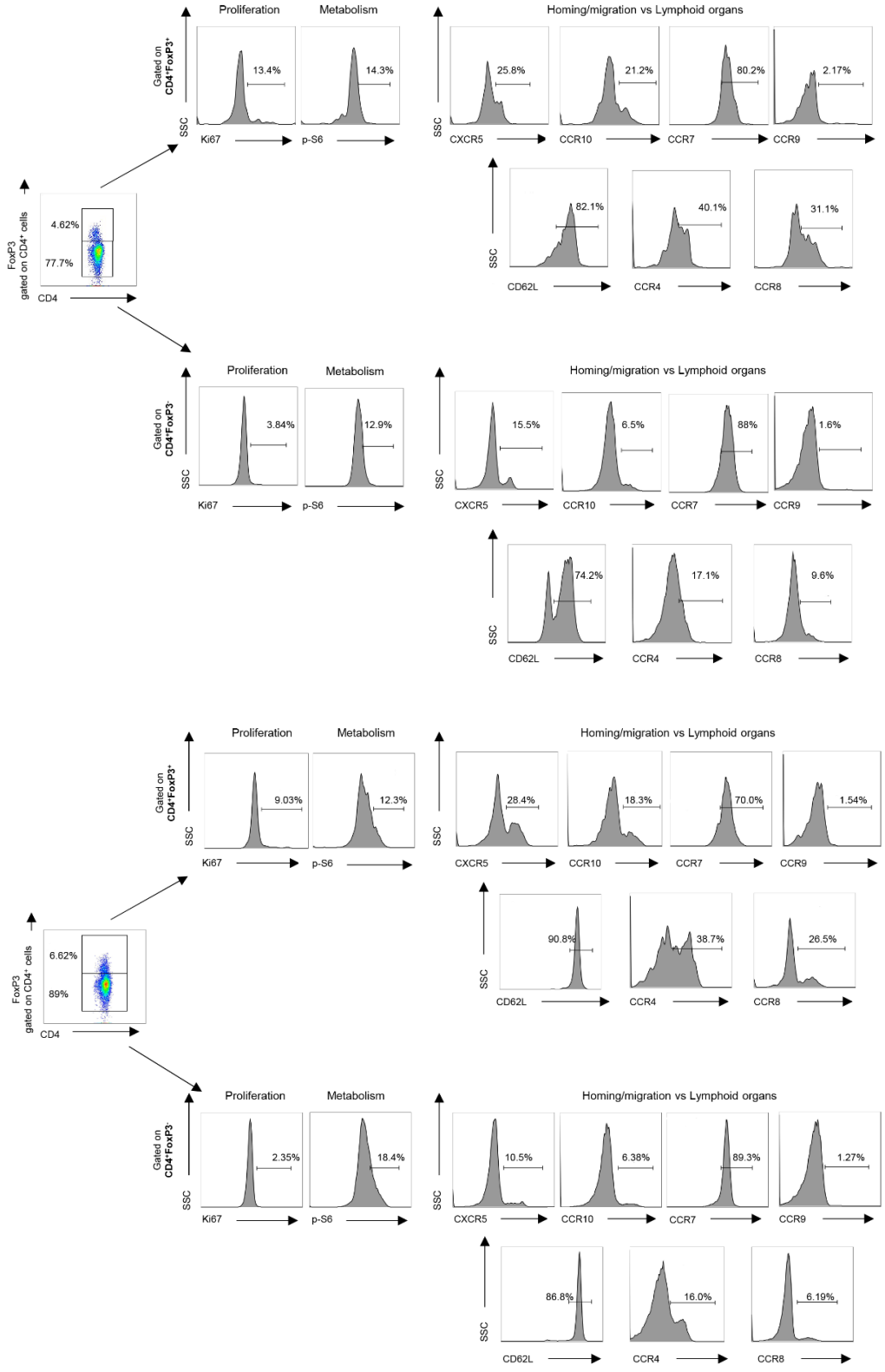


Fig. 15: Representative flow cytometry plots showing expression of Ki67, p-S6, CXCR5, CCR10, CCR7, CCR9, CD62L, CCR4 and CCR8 gated on $CD4^+FoxP3^+$ and $CD4^+FoxP3^-$ cells in PBMCs from RR-MS patients before (upper panels) and after 3 months of DMF treatment (lower panels).

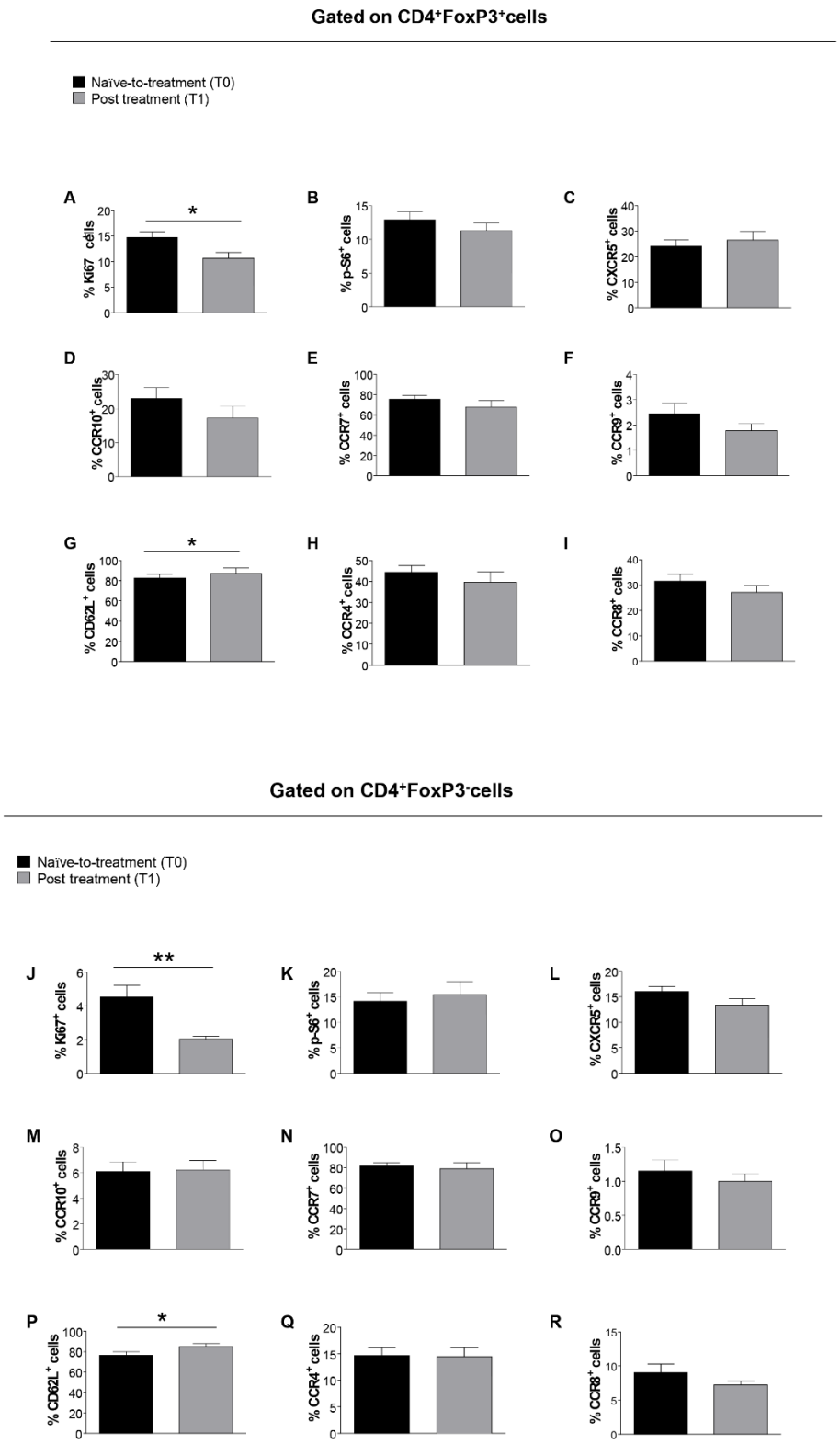


Fig. 16: Percentage of Ki67$^+$, p-S6$^+$, CXCR5$^+$, CCR10$^+$, CCR7$^+$, CCR9$^+$, CD62L$^+$, CCR4$^+$ and CCR8$^+$ cells gated on CD4$^+$FoxP3$^+$ cells (A-I) and gated on CD4$^+$FoxP3$^-$ cells (J-R) in freshly isolated PBMCs from RR-MS patients before (T0) and after 3 months of DMF treatment (T1). Data are shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$ by Mann - Whitney test.

4.6 DMF treatment increased the proliferative capacity of Treg cells in naïve-to-treatment RR-MS patients

Previous studies have shown that *in vitro* leptin neutralization with a neutralizing monoclonal antibody (mAb) during anti-CD3 and anti-CD28 stimulation, reversed Treg cell hyporesponsiveness and triggers their proliferation and secretion of IL-2 upon TCR stimulation [55]. In addition, it has been reported that Treg cells proliferation induced by leptin neutralization is impaired in patients with RR-MS [56]. To investigate whether DMF treatment could influence proliferation of Tconv and Treg cells, we isolated these subsets from naïve-to-treatment RR-MS patients and from patients switched to DMF from other therapies, before and after treatment with DMF. In naïve-to-treatment RR-MS patients, we found a trend towards an increase of Treg proliferation after leptin neutralization following two years of treatment **(Fig. 17A)**. We observed a similar trend also in RR-MS patients already treated with other drugs before the inclusion in the study. Indeed, we reported an increment of Treg cell proliferative capacity after 9 months of DMF treatment, even if after 24 months of DMF treatment Treg cell proliferation decreased to basal levels **(Fig. 17C)**. We next analyzed the proliferation of Tconv cells before and after treatment with DMF. We observed a significant increase of Tconv cell proliferation after DMF treatment in already treated patients **(Fig. 17D)** and only a trend to increased Tconv proliferation in naïve-to-treatment patients **(Fig. 17B)**.

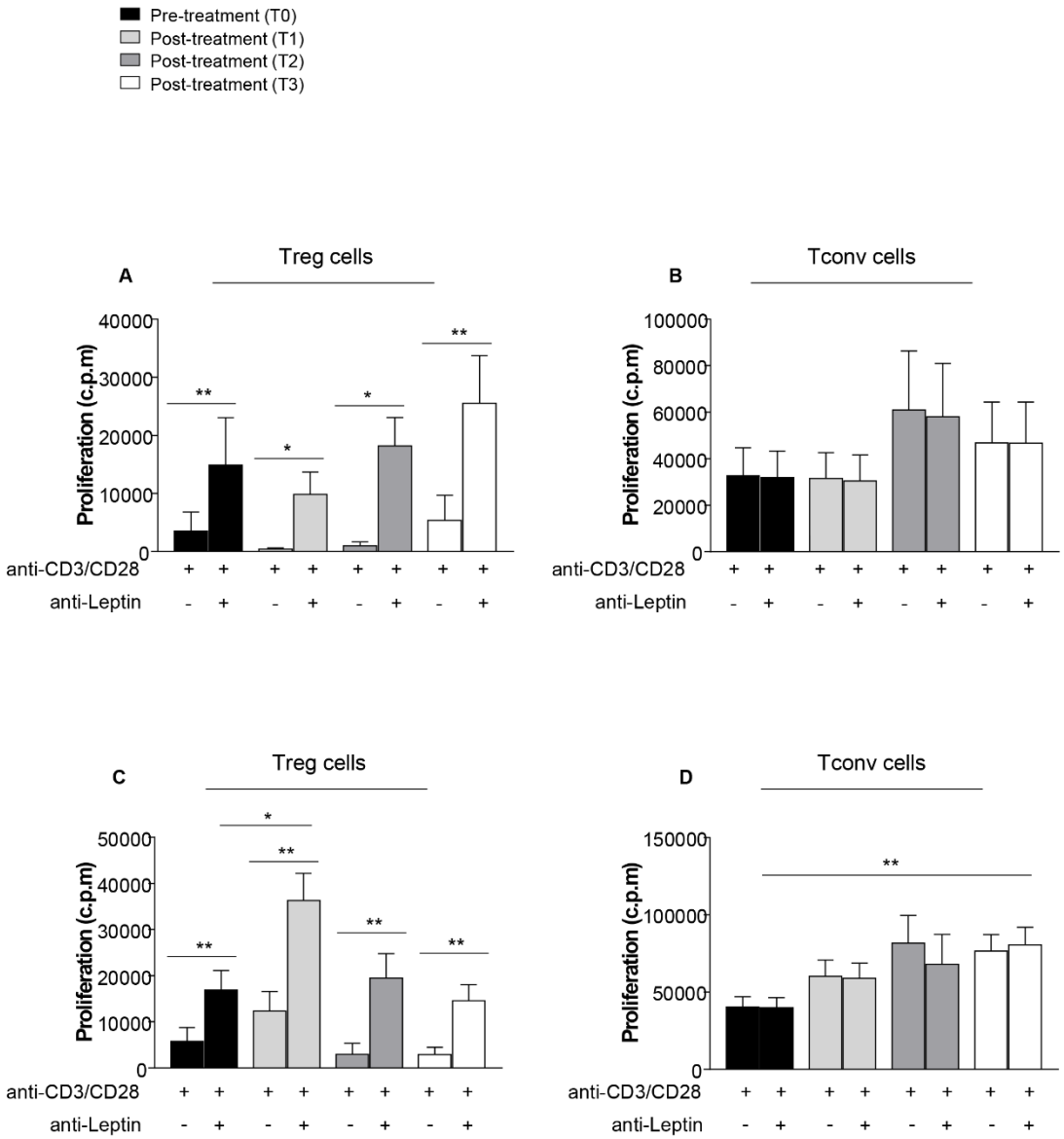


Fig. 17: Proliferation in the presence and absence of leptin neutralizing antibody of Treg and Tconv cells from naïve-to-treatment (upper panels) and switched from previous treatment RR-MS patients (lower panels) before (T0) and after 9, 18 and 24 months (T1, T2, T3) of DMF treatment. Data are shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$ by Wilcoxon matched-pairs signed rank test.

4.7 DMF treatment restored the altered metabolic profile of T cells in RR-MS patients

There is growing evidence showing how different metabolic pathways, in particular glycolysis, are involved in the induction and function of Treg cells in health and in autoimmunity [28]. For this reason, we performed functional analyses (Seahorse assays) to evaluate the extracellular acidification rate (ECAR), an indicator of aerobic glycolysis, and the O_2 consumption rate (OCR), an indicator of oxidative phosphorylation (OXPHOS), in PBMCs isolated from naïve-to-treatment RR-MS patients before and after 3 months of DMF treatment, upon T cell receptor (TCR) anti-CD3 stimulation (OKT3) **(Fig. 18)**. We observed that T cells from DMF treated RR-MS patients had an increased glycolytic metabolism after TCR stimulation when compared to T cells isolated from naïve-to-treatment counterpart **(Fig. 18A)** as testified by a sharp increase in ECAR levels evaluated as basal glycolysis, maximal ECAR (oligomycin-stimulated) and glycolytic capacity **(Fig. 18B-D)**. In parallel, to assess whether DMF treatment could influence mitochondrial functions, we measured OCR in basal conditions, after addition of oligomycin, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), and antimycin-A/rotenone (to inhibit electron transport chain, ETC) **(Fig. 18E)**. OCR was induced following DMF treatment compared to T cells isolated from naïve-to-treatment patients, as indicated by an increased maximal respiration, ATP-linked and spare capacity (ability to respond to ATP demand) **(Fig. 18F-H)**.

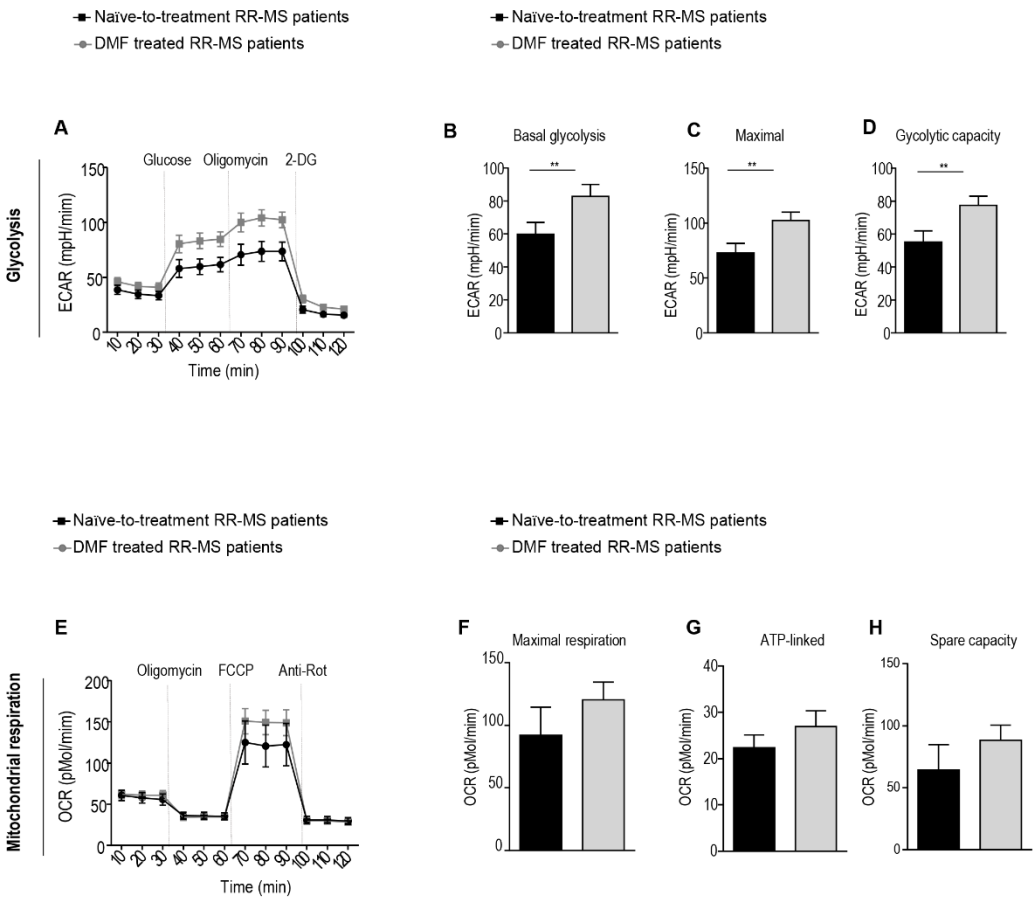


Fig. 18: (A) Kinetic profile of ECAR in PBMC from naïve-to-treatment and DMF treated RR-MS patients stimulated for 12hr with anti-CD3 (OKT3). ECAR was measured in real time, under basal conditions and in response to glucose, oligomycin and 2-deoxy-d-glucose (2-DG). Indices of glycolytic pathway activation, calculated from PBMC ECAR profile: basal glycolysis (B), maximal (C), and glycolytic capacity (D). Data are expressed as mean ± SEM. $^{**}p \leq 0.01$ by Wilcoxon matched-pairs signed rank test. (E) Kinetic profile of OCR in PBMC from naïve-to-treatment and DMF treated RR-MS patients stimulated for 12hr with anti-CD3 (OKT3). OCR was measured in real time, under basal conditions and in response to indicated mitochondrial inhibitors: oligomycin, FCCP, Antimycin A and Rotenone. Indices of mitochondrial respiratory function, calculated from PBMC OCR profile: maximal respiration (F), ATP-linked (G), and spare capacity (H).

5. Discussion

Multiple Sclerosis (MS) is an inflammatory and demyelinating disease that affects the central nervous system (CNS) and characterized by pathogenic T cell responses against myelin antigens followed by a broader neurodegenerative process [76]. Several effective treatments are available for patients with RR-MS, which are effective in reducing disease progression by restoring the balance of immune cells [77]. DMF is an oral drug approved as a first-line treatment for RR-MS patients [59-60]. Although several studies have been performed to understand the immunomodulatory effects of DMF, its mechanism of action is not yet fully understood.

In this study we firstly evaluated the effect of DMF treatment on the modulation of peripheral blood immune-phenotype in the context of MS.

We observed that after treatment with DMF, RR-MS patients showed a reduction in the number of lymphocytes in particular of $CD19^+$ B cells and $CD8^+$ and $CD4^+$ T cells in peripheral blood. Several studies showed that B cells may contribute to the development of MS pathogenesis. B and T cells are two major players in the pathogenesis of MS. It has been shown that B cells, together with production of antibodies, are also efficient antigen presenting cells and are able to process myelin antigens and subsequently activate pro-inflammatory T cells. This notion is supported by the immediate clinical benefit of therapeutic B cell depletion in MS, which acts probably by reducing the development of encephalitogenic T cells [78]. In agreement with previous studies [79-80], we observed that after treatment with DMF, RR-MS patients showed a reduction in the number of $CD19^+$ B cells in peripheral blood. This could be associated with a reduction of autoimmune reactions in CNS after DMF treatment. Our results show that also $CD8^+$ T lymphocytes are reduced during DMF treatment. These results are in agreement with other reports showing that DMF therapy modulates the frequency of $CD8^+$ T cells in the blood of MS patients [81-82].

This data is particularly interesting since it has been shown that $CD8^+$ T cells are involved in the pathophysiology of MS, in particular acting as potent effectors for CNS damage. Indeed, for decades, $CD4^+$ T cells have been considered the major players of disease pathogenesis. However, several evidences suggest that the pathological role of $CD8^+$ T cells in MS may have been underestimated as it has been shown that $CD8^+$ T cells could have a key role in the disease process [83].

Data from literature have shown that naïve-to-treatment RR-MS patients display a higher frequency of $CD4^+$ T cells with memory phenotype ($CD4^+CD45RO^+$) probably attributed to chronic inflammatory state leading to an increase in the memory population and a decrease in the pool of naïve T cells [84-85]. In addition, in MS patients, the majority of myelin basic protein (MBP)-reactive T cells resided in the memory T cell subset [86]. We observed that DMF treatment was able to reduce the subset of memory $CD4^+$ T cells that could be associated with a specific decrease of myelin-specific T cells. Depleting memory T cells may therefore be a key mechanism for DMF efficacy. These data are consistent with previous studies showing that DMF treatment shifts the immunophenotype of circulating lymphocyte subsets, inducing a significant reduction of memory cells [87-88].

We next investigated the mechanism underlying DMF-induced reduction of memory $CD4^+$ and $CD8^+$ T cells. We evaluated whether *in vivo* treatment with DMF might render circulating T cells more susceptible to apoptosis. We observed increased susceptibility of $CD4^+$ and $CD8^+$ T cells to apoptosis following DMF treatment. The analysis revealed that the rate of apoptosis induced by DMF treatment is higher in $CD4^+$ T cell with memory phenotype. This data could explain the reduction of $CD4^+$ T cells from memory compartment after DMF treatment. We next evaluated the level of different cytokines/chemokines/adipocytokines in plasma of RR-MS patients, before and after DMF treatment to understand whether the modulations of peripheral blood cell population levels could be attributable to concentration changes of serum molecules. We observed that DMF treatment was associated with a significant reduction of plasma resistin concentration only in RR-MS patients switched to DMF treatment after the enrolment in the study. Resistin, known as adipocyte-secreted factor (ADSF) or found in inflammatory zone 3 (FIZZ3), is secreted from immunocompetent cells, including macrophages and mononuclear cells in humans. Several lines of evidence support its engagement in inflammatory conditions *in vitro* as well as *in vivo* [89]. Serum levels of resistin were significantly higher in MS patients compared with healthy controls and correlate with circulating TNF-α, IL-1β and hs-CRP in MS patients suggesting that high level of this adipocytokine could enhance pro-inflammatory cytokine secretion in a positive feedback loop, which in turn leads to MS [90]. The reduction of resistin levels, after DMF treatment, could be also associated with reduction of other pro-inflammatory cytokines in MS patients. We also

evaluated the plasma levels of IL-8 (CXCL8) in RR-MS patients before and after DMF treatment. We observed a reduction of this chemokine overtime.

These data are particularly interesting since CXCL8 is a chemo-attractant molecule for both neutrophils and monocytes and triggers their firm adhesion to endothelium. In addition, serum CXCL8 and CXCL8 secretion from PBMCs were significantly higher in untreated MS patients compared to controls and this could be associated with a higher monocyte activity in MS together with increased monocyte recruitment to the CNS [91]. In addition, our analysis revealed a reduction, after DMF treatment, of plasma levels of MIP-1alpha (CCL3), a chemotactic chemokine secreted by macrophages. Since CCL3-stimulated migration of $CD4^+$ T cells was significantly increased in MS [92], the reduction of MIP-1alpa after treatment with DMF could be associated with a reduced activation and chemotaxis of leukocytes.

We next evaluated whether the reduction of circulating lymphoid cells, particularly T cells, after DMF treatment, was secondary to increased immune-redistribution and homing versus peripheral lymphoid organ and/or to modulation of cell proliferation. For this aim we evaluated the expression of CXCR5, CCR10, CCR7, CCR9, CD62L, CCR4, CCR8 and expression of the proliferation marker Ki67 by polychromatic FACS analysis on both Treg and Tconv cells. We found an increased expression of CD62L in both Treg and Tconv cells from RR-MS patients after DMF treatment. It has been reported that Treg cells are not a homogeneous cell population, but can be divided into two groups based on their surface expression of CD62L [93]. Although, both $CD62L^+$ and $CD62L^-$ subsets express FoxP3 and are anergic *in vitro*, the population that expresses CD62L is more proliferative and is characterized by greater suppressive function suggesting that the increased expression of CD62L after DMF treatment could be associated with a better suppressive function of Treg cells. Interestingly we found a reduction of proliferating Tconv cells after DMF treatment even if Ki67 expression is reduced also in Treg cells. The anergic state of Treg cells *in vitro* partly depends on their high metabolic rate, which is associated with the activation of the mammalian target of rapamycin (mTOR) pathway. It has previously reported that this nutrient-sensing metabolic pathway can be influenced directly by the adipokine leptin. Neutralization of leptin *in vitro* reverses Treg cell hyporesponsiveness and triggers their proliferation and secretion of IL-2 upon TCR stimulation while at the same time inhibiting Tconv cell proliferation and IL-2 production [56]. One of the immune

abnormalities observed in RR-MS patients is a reduction of the *in vitro* proliferative capacity of Treg cells after leptin neutralization.

This is associated with decreased expression of the transcription factor FoxP3 and with the overactivation of S6 ribosomal protein (a downstream target of mTOR) [56].

Therefore, we evaluated the effects of DMF treatment on the *in vitro* proliferative capacity of Treg cells. Here we found that, freshly isolated Treg cells from patients with RR-MS treated with DMF had increased their proliferation after incubation with a leptin-neutralizing monoclonal antibody (mAb). These data are particularly promising as the increased proliferative capacity of Treg cells may be associated with a higher control of peripheral tolerance and of autoimmune response occurring in patients with MS.

Several studies performed in the last few years have shown that cell metabolism is critical to regulate T cell fate since distinct metabolic programs may direct towards either effector or regulatory functions [94] and that important inhibitors of metabolism can regulate immune response in healthy and pathogenic cells [95]. Disturbed metabolic pathways could lead to altered functions of immune cells resulting in loss of immune tolerance to *self*-antigens [96]. Recent study reported that glycolysis was indispensable for the generation of human induced-Treg (iTreg) cells from Tconv cells through localization of the glycolytic enzyme enolase-1 to the nucleus, which directly affected the induction of specific splicing variants of FoxP3 containing the exon2 (FoxP3-E2) that were indispensable for the suppressive function of iTreg cells [28]. Moreover, the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), indicators of glycolysis and oxidative phosphorylation, respectively, were impaired during T cell activation in naïve-to-treatment RR-MS patients when compared with healthy controls [97].

Since the active form of DMF is fumarate, a TCA cycle metabolite, our hypothesis was that it may also act by altering T cell metabolism. Therefore, we also evaluated the effect of DMF treatment on glycolysis and mitochondrial respiration in T cells form RR-MS patients. We observed that DMF treatment was able to increase the glycolytic capacity of T cells from RR-MS patients. Since the engagement of glycolytic pathway is crucial for the generation and effector function of Treg cells, the increased glucose metabolism in T cell from DMF treated patients could be associated with a better control of autoimmune reactions.

6. Conclusion

In conclusion, our data suggest that DMF treatment is able to reduce the subset of memory $CD4^+$ and $CD8^+$ T cells and to increase the proliferative capacity of Treg cells over time. Furthermore, we observed that DMF treatment restores the altered metabolic profile of T cells in RR-MS patients. These data provide a novel mechanism of action for DMF through modulation of the metabolic pathways of T cells that could lead to an improvement in immune tolerance during autoimmunity.

7. References

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