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# UNRAVELLING THE ROLE OF MUCOSAL ASSOCIATED INVARIANT T CELLS IN THE PATHOGENESIS OF MULTIPLE SCLEROSIS AND ITS ANIMAL MODEL

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## Abstract

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS), characterized by demyelination and axonal loss. It's considered a multifactorial disease, whose etiology involves both environmental and genetic factors. The innate and the adaptive immune systems have long been considered the main players in CNS inflammation, plaque formation and, ultimately, neurodegeneration. In the last 10 to 15 years a lot of attention has been focused on a newly described subset of immune cells, called unconventional T cells, as possible modulators of the aberrant activation of the immune system. The family of unconventional T cells comprises mucosal associated invariant T (MAIT), $\gamma\delta$ and NKT cells. MAIT cells are innate-like T cells, recognizing riboflavin metabolites presented by the evolutionary conserved antigen presenting molecule MR1. They are abundant at mucosal sites and their phenotype is deeply influenced by the microbiome. They can be activated either by direct TCR stimulation or by cytokines produced during inflammation. Once activated, they can produce both pro-inflammatory cytokines and cytotoxic molecule, like granzyme B. MAIT cells have been implicated in the pathogenesis of many autoimmune diseases, such as diabetes, inflammatory bowel diseases and rheumatoid arthritis. In this project we aim to elucidate the potential role of MAIT cells in the pathogenesis of MS, by using both the animal model of the disease, experimental autoimmune encephalomyelitis (EAE) and samples obtained from people with MS (pwMS).

# Introduction

## Multiple Sclerosis

Multiple Sclerosis is an autoimmune demyelinating disease of the central nervous system (CNS). It involves demyelination, inflammation, and varying degree of axonal degeneration. MS affects 2.8 million people worldwide, with significant personal and socioeconomic burden. Clinically, MS can be relapsing remitting (RRMS), or can have a progressive course characterized by accumulating neurological disability with or without superimposed relapse activity. Genetic risk factors do not account fully for disease development. Environmental factors including some infections, low vitamin D levels, smoking and obesity have each been associated with increased MS risk. Both the innate and the adaptive immune system have been implicated in disease pathogenesis, even if their exact role has not been completely elucidated.

### Epidemiology

The prevalence of MS varies from 33 to 133 per 100,000 worldwide, with the highest prevalence in Western Europe and North America [1]. Several studies have shown the existence of a latitude gradient[2], with people living in countries closer to the equator being at lower risk of MS, whilst those living in countries at higher latitudes (closer to the north/south poles) being at greater risk (**Figure 1**).

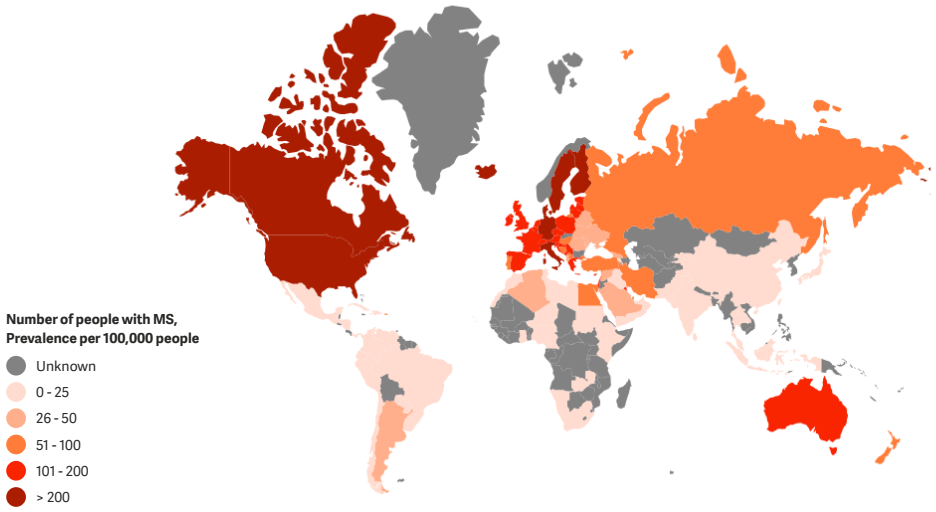


**Figure 1**. **World prevalence of MS.** From *www.atlasofms.org*

MS is twice as common among women than men. This skew towards females is even greater in the Western Pacific and South-East Asia regions where there are more than three times as many females to males[1]. The reasons for the difference in risk between males and females are unknown but a variety of factors are likely to be influencing it such as hormonal[3] and genetic differences[4], as well as differing social, lifestyle and environmental exposures between the sexes.

MS can occur at any age, but the average age at MS diagnosis globally is 32 years, making it the most common cause of neurological disability in people younger than 65 years[1]. The average age of diagnosis is relatively consistent across the regions of the world (ranging from 30-33 years across the six WHO regions). However, there is greater variation in the average age of diagnosis across individual countries, ranging from 20 years up to 50 years[1]. MS not only affects adults. It is estimated than 1.5% of people diagnosed with MS every year are below the age of 18[1].

## Etiology

MS is considered a multifactorial disease, resulting from the addictive interaction between different classes of risk factors. A combined analysis of both prominent genetic and environmental risk factors showed that a major fraction of MS risk could be explained by currently known risk factors[5].

### *Environmental risk factors*

Established MS-associated environmental risk factors include smoking, low vitamin D levels caused by insufficient sun exposure and/or dietary intake, childhood obesity and Epstein–Barr virus (EBV) infection[6,7]. Smoking and MS risk have a dose–response relationship: cumulative smoking is associated with an increase in the risk[8]. Moreover, smoking interacts with MS-associated HLA-variant risk alleles, contributing to an increased risk of developing the disease in genetically predisposed individuals[9].

Many infectious agents have been proposed to have a role in MS, but one of the most interesting candidate viruses is EBV. People who have had clinically overt infectious mononucleosis have a >2-fold increased risk of developing MS. Several hypotheses have been proposed to explain how EBV infection may play a role in MS pathophysiology. In the periphery, EBV may contribute to aberrant activation and trafficking of CNS-reactive immune cells, resulting in disease relapses. The molecular mimicry theory describes how T cells primed by exposure to EBV antigens cross

react to recognize and attack CNS antigens[10]. Within the CNS, EBV may contribute to propagating target organ inflammation and injury. This is supported by the idea that EBV could elicit "bystander damage" by inducing an antiviral immune response against infected cells in the CNS[11]. Low vitamin D levels, in particular before the age of 20, have been associated with an increased risk of developing MS in a pioneering study by Munger et al.[12]. Since then, many studies have tried to explain the exact link between vitamin D exposure and MS risk, with inconsistent results[13–15]. However it's now accepted that vitamin D and/ or sun exposure in adolescence is an important modulator of future MS risk[16].

Obesity, in particular during childhood and adolescence, has been recently associated with an increased risk of developing MS. Large cohort studies have associated obesity during adolescence with future risk of MS in females[17–19] and, more recently, mendelian randomization studies show that genetic determinants for high BMI are associated with increased MS risk[20,21]. Also in this case, the mechanism through which an increase in BMI is linked to and increased risk of developing MS is not clear. Both a pro-inflammatory state and changes in gut microbiome composition have been proposed as link between higher adiposity and neuroinflammation, but without definite conclusions[16].

### *Genetic risk factors*

Genes within the HLA complex are the strongest genetic risk factors for MS. The HLA class II and I genes are particularly relevant modifiers of disease risk: variants of class II genes encode products that present antigens to $CD4^+$ T lymphocytes, and class I products present antigens to $CD8^+$ lymphocytes. In MS, the class II variant HLA-DRB1*15:01 has a striking association with an increased risk of MS (odds ratio (OR) ~3), whereas the class I variant HLA-A*02 is associated with protection from the disease (OR ~0.6). The absence of HLA-A*02 and the presence of DRB1*15:01 has a combined OR of ~5[22–24]. Genome-wide association studies (GWAS) have detected ~110 non-HLA single nucleotide polymorphisms (SNPs), each of which confers a modest influence on MS risk; ongoing large GWAS are expected to identify more such SNPs. Nearly all of the known MS-associated SNPs are located close to genes and regulate either adaptive or innate immunity, providing further evidence that MS is primarily an immune mediated disease[16,25,26].

## Pathogenesis

The pathogenesis of MS is mediated primarily by T cells[27,28]. T cells are most likely primed to CNS autoantigens in the periphery and then cross the blood–brain barrier to activate microglia and macrophages. In concert, these cells induce the death of myelin-producing oligodendrocytes and directly damage the myelin sheath around nerve fibers to generate active lesions in the CNS.

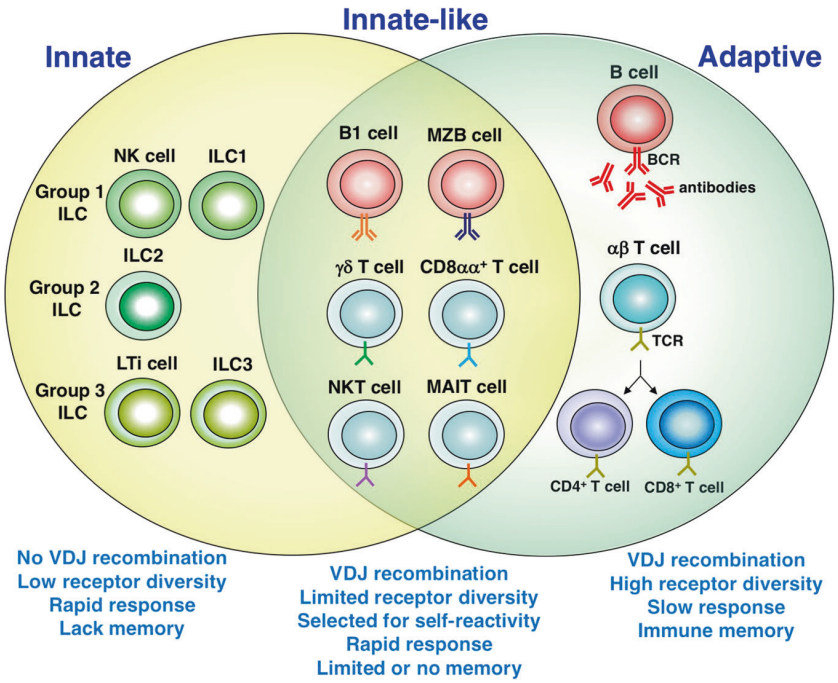


**Figure 2**. **Subsets of innate, innate-like, and adaptive lymphocytes involved in the pathogenesis of MS**. From *Kaer et al., 2019*[33]

The key role of T cells, particularly $CD4^+$ T cells, in MS has been confirmed in experimental autoimmune encephalomyelitis (EAE), which is the main animal model of MS and can be induced in a variety of mammalian species through immunization with myelin antigens accompanied by adjuvant or through the adoptive transfer of myelin-reactive T cells[29]. However, many other lymphocyte subsets have been implicated in disease pathogenesis and these subsets include lymphocytes that belong to the adaptive immune system, to the innate immune system, and innate-like B and T lymphocytes that exhibit properties of both innate and adaptive immunity[30,31] (**Figure 2**).

## *Innate immunity*

### Innate lymphocytes

It is now appreciated that the innate immune system includes a variety of lymphoid subsets, called innate lymphoid cells (ILCs), which are strategically positioned in many tissues of the body to provide protection against infections and to control tissue inflammation[32]. ILCs have been divided into three distinct groups based on their expression of transcription factors, cytokine profiles, and functional properties. Group 1 ILCs include classical natural killer (NK) cells and ILC1 cells that produce IFN-γ, group 2 ILCs include ILC2 cells that produce IL-4, IL-5, and IL-13, and group 3 ILCs include lymphoid tissue inducer (LTi) cells implicated in the development of secondary lymphoid organs and ILC3 cells that produce IL-17 and IL-22[33]. These cells generally have roles very early in the immune system, are highly responsive to activation by certain cytokines, and can interact with adaptive lymphocytes[34].

NK cells are innate lymphocytes with cytolytic activity, and they can produce the pro-inflammatory cytokines IFN-γ and TNF-α, the anti-inflammatory cytokine IL-10, and the growth factor GM-CSF in response to activation by cytokines such as IL-12, IL-15, and IL-18[35]. In humans (but not in mice), NK cells can be distinguished by the expression of CD56, a neural cell adhesion molecule[36]. Several studies focused on the possible role of NK cells in the pathogenesis of MS, with contrasting results. In the CSF of MS patients, the $CD56^{bright}$ population is expanded compared with the $CD56^{dim}$ population[37]. Several therapies, approved for the treatment of MS, induce an increase in the $CD56^{bright}$ NK cell population in the circulation of patients[38,39]. These findings are consistent with the suppressive effect of the $CD56^{bright}$ NK cell population observed in MS[40]. In EAE, the depletion of NK cells has resulted in contradictory results: some studies have provided evidence for disease exacerbation[41–43] and other studies have demonstrated that NK cell depletion results in disease amelioration[44].

LTi cells are a subset of ILCs that are critically important for the development of secondary lymphoid organs[45]. These cells also control the generation of organized follicle-like structures called tertiary lymphoid follicles that might develop during chronic inflammation in tissues, including the CNS[46,47]. Several studies have provided evidence showing that LTi cells are

expanded in the CNS and circulation of patients with MS[48,49], which suggests that these cells have a pathogenic role by promoting the generation of tertiary lymphoid follicles[50].

### Monocytes and Microglia

Monocytes mainly enter the CNS as part of an acute inflammatory response, whereas microglial cells arise from primitive myeloid progenitors in the yolk sac and migrate to the CNS during early embryonic development[51]. The population of microglial cells is maintained by self-renewal with little, if any, contribution from the blood. Microglial cells play essential parts, not only during CNS inflammation, but also during CNS homoeostasis[52].

Mononuclear phagocytes, such as microglia and macrophages, are the dominant immune cells located in multiple sclerosis lesions in both relapsing–remitting and progressive phases of the disease[53]. These cells interact with cells of the adaptive immune system but can also directly cause neuroinflammatory tissue damage. Phagocytes are mainly responsible for myelin damage and removal, the hallmark of multiple sclerosis lesions, and the level of activity in these lesions can be staged by the presence of myelin degradation products in phagocytes[54]. However phagocytes are also found close to damaged axons[55] and the number of these cells is positively associated with the extent of acute axonal damage in a multiple sclerosis lesion[56].

Moreover, studies[57,58] using animal models of CNS injury suggested that these cells might also be a necessary part of the tissue repair mechanism during lesion resolution.

### *Adaptive immunity*

Both $CD4^+$ and $CD8^+$ T cells are found in the brain lesions of pwMS[59,60]. In the CNS, $CD4^+$ T cells recognize antigens presented by microglia cells, infiltrating macrophages, and B cells, all expressing HLA class II molecules. By contrast, $CD8^+$ T cells recognize antigens presented by HLA class I molecules, which are expressed on all nucleated cells, including oligodendrocytes, astrocytes, and neurons. $CD4^+$ T cells have a critical role in the pathogenesis of EAE. The susceptibility of different mouse strains to EAE is correlated with their major histocompatibility complex (MHC) haplotype. The adoptive transfer of $CD4^+$ T cells from mice immunized with myelin antigens can often induce disease in recipient animals[61]. The adoptive transfer of myelin antigen-specific $CD8^+$ T cells in the EAE model induces disease in the recipient animals and causes CNS lesions and symptoms that are more similar to those found in human disease than those

observed in $CD4^+$ T-cell-mediated EAE[62]. Results from analyses of T-cell receptors showed that substantial fractions of $CD4^+$ T cells and $CD8^+$ T cells in both MS and the EAE animal model, derived from clonal expansion, suggesting that antigen-specific T-cell responses contribute to the disease process[63,64]. Although clonally expanded T cells in the CNS parenchyma of patients with multiple sclerosis could target CNS autoantigens, these T cells could also possibly be long-lived resident memory T cells directed against common neurotropic viruses that would reside in the CNS and expand due to specific cytokine stimuli independently of their primary antigen[65]. Despite efforts to identify T-cell target antigens in multiple sclerosis[64,66] the epitopes recognized by T cells in the perivascular cuffs or in the CNS parenchyma remain unknown. In the CSF, most $CD4^+$ T cells have a central memory phenotype[53] (ie, T cells express CD45RO and CCR7) and can produce interferon γ. Fewer $CD4^+$ T cells in the CSF produce interleukin 17 and their proportion seems to be higher in patients in acute relapse than in those patients with multiple sclerosis with no disease activity[67].

B cells, plasmablasts, and plasma cells are present in lesions, meninges, and the CSF in most patients with multiple sclerosis[68]. B cells and plasma cells in the CNS are likely to be supported by cytokines and survival factors that are, at least partly, produced by glial cells[68]. Findings from immunohistochemistry and flow cytometry studies on B cells and plasma cells in the meninges and CSF suggested an association between the presence of these cells, disease activity, and the clinical outcome[69]. Similarly, the presence of oligoclonal bands in patients with a clinically isolated syndrome is associated with an increased risk of conversion to multiple sclerosis[70].

In EAE, B cells have distinct pathogenic or regulatory functions, depending on the stage of disease[71]. B-cell-deficient mice exhibit an exacerbated disease course, which is due to the regulatory functions of B cells and their capacity to produce IL-10[72]. B-cell depletion using anti-CD20 antibodies before EAE induction causes exacerbated disease and this effect is associated with the loss of IL-10-producing Bregs[73]. However, B-cell depletion after EAE induction ameliorates disease severity due to the pathogenic role of memory B cells, which facilitate T-cell reactivation during the later disease stages[73].

*Innate-like lymphocytes*

Unconventional, or innate-like T cells, are non-MHC restricted lymphocytes that recognize non-polymorphic antigen-presenting molecules. Some of these are encoded by genes outside the MHC locus (CD1a, CD1b, CD1c, CD1d and MR1) or within the MHC locus (HLA-E, HFE, H2-M3 and H2-Q9). The responding T cells are considered 'unconventional' because they do not recognize classical peptide antigens; they are not donor restricted, and they circulate as abundant populations of cells that are poised for rapid response[74] (**Figure 3**).

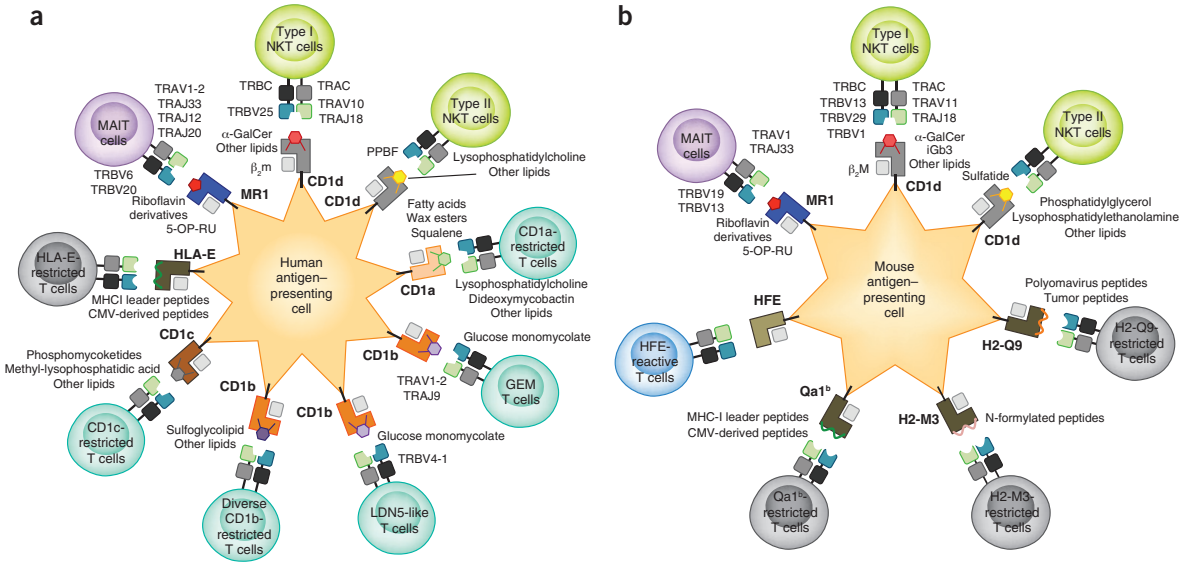


**Figure 3**. **Unconventional αβ T cells in human (a) and mouse (b)**. From *Godfrey et al., 2015*[78]

γδ T cells represent 0.5–10% of circulating lymphocytes in adult humans and mice[74]. γδ T cells generate TCR heterodimers through somatic recombination of genes encoding TCR variable (V), diversity (D), joining (J) and constant (C) regions. The number of V, D and J segments is more limited for γδ T cells than for αβ T cells, and many γδ T cells use germline gene rearrangements or are predisposed to specific TCRγ- and TCRδ-chain pairings[75]. γδ T cells have been found in MS lesions in the brain and CSF of patients with recent onset disease, where they are clonally expanded, which is suggestive of antigenic stimulation[76]. In mice, γδ T cells can be found in the CNS during steady-state conditions and their number increases during the development of EAE[77]. To further support their possible pathogenic role in EAE, studies demonstrated that the depletion

of γδ T cells using monoclonal antibodies suppresses EAE severity[78] and mice that are genetically deficient in γδ T cells are protected against EAE[79].

NKT cells are $CD3^+$ T cells that recognize lipid antigens presented by the MHC class I-related protein CD1d[80]. Two broad types of CD1d-restricted NKT cells exist: type I NKT cells, sometimes referred to as 'invariant NKT cells' or 'iNKT cells', which express an invariant TCRα chain (TRAV11-TRAJ18 in mice and TRAV10 and TRAJ18 in humans) and a limited, but not invariant, range of TCRβ chains; type II NKT cells, also known as 'diverse NKT cells' because they use more diverse or oligoclonal αβ TCRs that do not conform to the TCR motifs described above[80]. Type I NKT cells can be classified based on their cytokine profile: mouse studies have defined IFN-γ producing T-$bet^+$ NKT1 cells, IL-4-producing GATA-$3^+$ NKT2 cells, IL-10-producing NKT10 cells, IL-17-producing RORγτ$^+$ NKT17 cells and IL-21-producing Bcl-$6^+$ NKT-FH (follicular helper) cells[74]. The numbers and functional activities of iNKT cells are decreased in the circulation of MS patients[81]. In patients in remission in the absence of treatment iNKT cells adopt a Th2-biased cytokine production profile, which suggests potential suppressive activities against the disease process[82].

EAE studies with NKT cell-deficient mice have provided divergent results. In CD1d-deficient mice, which lack both iNKT and dNKT cells, either no effect or disease exacerbation has been observed[83,84]. Vα14-Jα18 TCR transgenic mice that overproduce iNKT cells are partially protected against EAE through a mechanism that involves iNKT cell infiltration into the CNS and inhibition of autoantigen-specific Th cell responses in the spleen[85].

### MAIT cells

MAIT cells recognize riboflavin metabolites presented by the MHC class-I related protein MR1. MR1, a non-polymorphic, MHC class I–like protein, governs the development and function of MAIT cells[86]. MAIT cells typically express an invariant TCRα chain consisting of TRAV1-2 joined to TRAJ33 in humans and mice, with a constrained TCRβ-chain repertoire (TRBV19 and TRBV13 in mice, and TRBV6 and TRBV20 in humans)[87]. MAIT cells are predominantly $CD8^+$ or $CD4^-CD8^-$ in mice and humans, even if $CD4^+$ MAIT cells exist in both species[74]. While MAIT cells are generally low in frequency in laboratory mice[88], they are abundant in humans, representing on average approximately 5% of total blood T cells, 10% of $CD8^+$ T cells, and up to 45% of liver T cells[89,90]. MAIT cells respond to many strains of bacteria and yeast but not viruses[89]. MR1 ligands include

derivatives of vitamin B9 (folate), and relatively unstable pyrimidine intermediates derived from the vitamin B2 (riboflavin)–synthesis pathway. While folate derivatives, such as 6-FP (6-formylpterin) can inhibit the activation of MAIT cells, derivatives of the riboflavin pathway, such as 5-OP-RU (5-(2-oxopropylideneamino)-6-d-ribitylaminouracil), potently activate MAIT cells[91,92]. The microbial species that activate MAIT cells all have an intact riboflavin pathway, whereas organisms that do not activate MAIT cells lack this pathway[91].

MAIT cells are present in low number and with a naïve phenotype in human cord blood. They are thought to expand and mature upon exposure to microbial-derived antigens[93–95]. In mice, it has been demonstrated they acquire their final phenotype in the first weeks of life, deeply influenced by the gut microbiota[95]. Upon activation, MAIT cells are almost the exclusive IL17 producers of all $CD8^+$ T cells, which is in accordance with the high expression of CD161 and CCR6[90]. MAIT cells also secrete IFN-γ, TNF-α, perforin and granzyme B[90].

MAIT cells activation is different when mucosal cells are compared to circulating cells. For example, intestinal derived MAIT cells are more activated (higher expression of CD69, HLA-DR, and CD25), but they also express higher levels of inhibitory receptors, such as TIGIT, CTLA-4, PD1, and LAG3[96]. This phenotype might reflect continuous exposure to metabolites derived from commensals and/or pathogenic bacteria[97]. During bacterial infections, in addition to MR1-antigen complexes, MAIT cells are exposed to a variety of inflammatory cytokines that co-stimulate and enhance their activation, potentially overcoming inhibitory signals[97]. The relative importance of TCR-driven vs. cytokine driven MAIT cell stimulation also changes during the course of an infection, with the former dominating at earlier stages of the response[98].

There is ongoing controversy about the involvement of MAIT cells in the pathogenesis of MS. MAIT cells. The antigen presenting molecule MR1 have been detected in MS lesions but only low numbers of MAIT cells were detected in the CSF of pwMS[99,100]. Diverging results on circulating MAIT cell frequencies in pwMS compared with healthy controls were reported[100–102]. Blood MAIT cell frequencies are influenced by smoking behavior, which could possibly have influenced frequencies of circulating MAIT cells in previous studies[103]. Finally, few studies have reported an impaired cytokine production after non-specific in vitro stimulation of MAIT cells obtained from pwMS compared with controls[102,104].

Because of their very low number in mice, studies focusing on the role of MAIT cells during EAE are scarce. In an elegant study by Croxford and collaborators from 2006, MAIT cells seem to have a protective role against the development of the disease[105]. The disease is suppressed in Vα19i transgenic mice, expressing high numbers of MAIT cells[105]. The protection is accompanied by a higher production of the immunoregulatory cytokine IL-10[105].

### Clinical presentation and diagnosis

Symptoms of MS result from interruption of myelinated tracts in CNS, are extremely varied and depend on the location and severity of lesions within the CNS. At the beginning of the disease, signs and symptoms include more frequently: sensory disturbances (30.7%), visual alterations (15.9%), diplopia (6.8%), ataxia (4.8%), pyramidal system disorders, both acute (4.3%) and subacute (8.9%). A few symptoms and signs are particularly pertinent to MS, especially in combination with a suitable clinical history. These include Lhermitte's phenomenon, which is precipitated by neck flexion and is characterized by brief electric shock sensations in the spine, legs, or arms. Examination of eye movements may reveal internuclear ophthalmoplegia, which is almost pathognomonic of MS. Longstanding optic atrophy can be visualized on fundoscopy and commonly causes a relative afferent pupillary defect in the same eye. This may accompany a change in color vision or acuity, or indeed a central scotoma. Some patients experience Uhthoff's phenomenon, in which their symptoms become transiently exacerbated by hot weather or exercise; this relates directly to diminution in nerve conduction in partially demyelinated nerve fibers at higher temperatures. Many patients describe fatigue, difficulties in jumping, running or going up or down the stairs[106]. Furthermore, some patients experience recurring, brief, stereotypical phenomena highly suggestive of MS, such as paroxysmal pain or paresthesia – thought to represent discharges originating along demyelinated axons –, trigeminal neuralgia, episodic clumsiness or dysarthria, and tonic limb posturing. Depression and cognitive deficits are common, especially in advanced cases; cognitive deficits include memory loss, impaired attention, problem-solving difficulties, slowed information processing, and difficulties in shifting between cognitive tasks[107].

The course of multiple sclerosis in an individual patient is largely unpredictable. According to the 2013 revisions of 1996 standardized descriptions for MS phenotypes[108] the pattern and course

of MS is categorized into four clinical subtypes: clinically isolated syndromes (CIS), relapsing-remitting MS (RR-MS), secondary progressive MS (SP-MS) and primary progressive MS (PP-MS):

- Clinically isolated syndrome (CIS): the first clinical presentation of a disease that shows characteristics of inflammatory demyelination that could be MS but has yet to fulfill criteria of dissemination in time. A CIS is, by definition, always isolated in time (monophasic) and, usually also in space (monofocal).
- Relapsing-remitting multiple sclerosis (RR-MS) accounts for 85% of MS cases at onset and is characterized by unpredictable attacks (relapses, exacerbation) followed by periods of months to years of relative quiet condition with no new signs of disease activity. Relapses are thought to represent recurrent episodes of inflammation and demyelination often accompanied by axonal injury that occur often acutely or subacutely, with symptoms developing over hours (lasting for at least 24 hours, in the absence of fever or infection)[109] to several days, persisting for days to several weeks, and then gradually dissipating. Most of the time they improve, spontaneously or in response to high-dose steroids, in weeks. Regression of symptoms has been attributed to the resolution of inflammation and edema, to spontaneous remyelination, to the proliferation, migration, and differentiation of resident oligodendrocyte precursor cells and to spread of sodium channel from the nodes of Ranvier to cover denuded axon segments and restore conduction. The outcome in RR-MS is variable: untreated, about 50% of all MS patients require at least a walking aid within 10 years after clinical onset.
- Secondary progressive multiple sclerosis (SP-MS): around 60–70% of RRMS patients transform into a secondary-progressive disease course (SPMS) within 20-25 years from disease onset80. In most clinical contexts, SPMS is diagnosed retrospectively by a history of gradual worsening after an initial relapsing disease course, with or without acute exacerbations during the progressive course[108].
- Primary progressive multiple sclerosis (PP-MS) constitutes 10% of all cases[1]. These patients do not experience attacks but have only a continuous and steady worsening of neurologic functions from the onset of disease. Compared to RRMS the gender distinction is less strong. The disease begins later in life (mean age 40 years) and disability develops

faster. Clinically, this form of the disease is associated with a lack of response to any form of immunotherapy.

In addition, these phenotypes are further defined by assessments of disease activity and disease progression. Disease activity is determined by clinical relapses or MRI evidence of contrast enhancing lesions and/or new or unequivocally enlarging T2 lesions. Disease progression is a process that is independently quantified from relapses, and is characteristic of primary and secondary progressive MS. The term progressive-relapsing MS (PR-MS)[110] previously used to characterize patients who had progressive disease from onset and clear acute relapses, is now obsolete. An acute attack in a patient with progressive disease from onset is now considered to be PP-MS with active disease[108].

Radiologically isolated syndrome (RIS) represents the incidental finding of typical MS lesions on MRI[108], but is not considered a separate MS phenotype, since such patients lack clinical signs and symptoms of the disease. Follow-up studies done over 2–5 years have independently reported that about 30–40% of such people have one or more clinical events leading to a diagnosis of CIS or MS[109]. This group can thus be thought to be at high risk for MS.

The diagnosis of MS is primarily clinical and relies on the demonstration of symptoms and signs attributable to white matter lesions that are disseminated in time (DIT) and disseminated in space (DIS), along with the exclusion of other condition that may mimic MS. Diagnostic criteria for MS, developed in the early 1980s (the Poser criteria), considered clinical characteristics and a number of laboratory studies including cerebrospinal fluid analysis, evoked potentials, and neuroimaging[111]. They have been later supplanted by the McDonald criteria, which were developed in 2001[112] and subsequently revised in 2005[113], 2010[114] and 2017[115] in order to incorporate newer evidences and to simplify the use of neuroimaging while preserving the sensitivity and specificity of the criteria.

The most recent 2017 McDonald criteria focus on the integration of clinical course, laboratory findings, and MRI data to establish a diagnosis of MS (**Table 1**)

| | Number of lesions with objective clinical evidence | Additional data needed for a diagnosis of multiple sclerosis |
|---|---|---|
| **≥2 clinical attacks** | ≥2 | None |
| **≥2 clinical attacks** | 1 | None |
| **≥2 clinical attacks** | 1 | DIS demonstrated by an additional clinical attack implicating a different CNS site or by MRI |
| **1 clinical attack** | ≥2 | DIT demonstrated by an additional clinical attack or by MRI OR demonstration of CSF-specific OCBs |
| **1 clinical attack** | 1 | DIS demonstrated by an additional clinical attack implicating a different CNS site or by MRI and DIT demonstrated by an additional clinical attack or by MRI or demonstration of CSF-specific OCBs |

**Table 1**. **Diagnostic criteria for the diagnosis of MS**. From *Thompson et al., 2018*[115]

For patients who present with insidious neurological progression suggestive of PPMS, the criteria require evidence of the one year of disease progression (retrospectively or prospectively determined) plus two of the three following criteria: 1) Evidence for DIS in the brain based on ≥1 T2-lesions in at least 1 area characteristic for MS (periventricular, juxtacortical, or infratentorial); 2) Evidence for DIS in the spinal cord based on ≥2 T2-lesions in the cord; 3) Positive CSF findings (evidence of OB and/or elevated IgG index)[115].

## Therapy

All patients with a diagnosis of RR-MS and SP-MS are candidates for treatment. The target of the current treatments is the immune response and the neuroinflammation, which predominate during the initial phase of the disease and decrease over time. On the contrary, these drugs haven't much influence on secondary signaling changes in astrocytes and neurons which contribute to neurodegeneration and constant disease progression[116]. Therefore, it is recommended to initiate treatment as soon as possible.

First line therapy for an acute attack (relapse) are corticosteroids. Indications for treatment include functionally disabling symptoms with objective evidence of neurologic impairment (e.g., loss of vision, motor, and/or cerebellar symptoms). Mild sensory attacks are often not treated in the same manner, although symptomatic relief is sometimes necessary because of patient discomfort (e.g., due to paresthesia). It has been proven that corticosteroid therapy shortens the duration of the relapse and accelerates recovery[117]. Intravenous corticosteroid treatment is ineffective in ~15% of cases[118]. The second-line therapy after corticosteroid failure or unresponsiveness is plasma exchange (PLEX)[118].

Immunomodulatory agents (the so-called disease-modifying treatments - DMTs) have become the cornerstone of treatment for patients with RR-MS and SP-MS, with the aim of avoiding new relapses and controlling CNS inflammatory state. Nowadays there are twelve immunomodulatory drugs (beta-interferon compounds, glatiramer acetate, ofatumumab, natalizumab, fingolimod, siponimod, ponesimod, ozanimod, teriflunomide, dimethyl-fumarate, monomethyl-fumarate, diroximel-fumarate) and four immunosuppressive drugs approved by the FDA (cladribine, mitoxantrone, alemtuzumab and ocrelizumab).

There are two opposite schemes of treatments for MS: the escalating approach and the induction therapy. The rationale of escalating therapy is to start treatment with safe drugs and to move to more aggressive treatments only in case of failure of the on-going treatment[119]. Patients start with the first line drugs (such as GA and interferons), then move to second-line drugs (such as fingolimod and natalizumab) or immunosuppressive agents (cyclophosphamide and mitoxantrone). On the other hand, the induction strategy uses early and aggressive immunotherapy in hopes of postponing or preventing the latter outcome. Once in sustained remission, patients could then transition to “safer” immunomodulatory therapies, reasoning that the more aggressive therapy might provoke a long-term immunological “reset” with benefits[119].

## Experimental Autoimmune Encephalomyelitis

Our understanding of the inflammatory response in multiple sclerosis has been greatly shaped by findings from animal models of the disease such as experimental autoimmune encephalomyelitis (EAE)[120].

Inflammatory demyelination in experimental autoimmune encephalomyelitis is dependent on $CD4^+$ T cells that react to myelin proteins, including myelin basic protein and myelin oligodendrocyte glycoprotein (MOG)[121]. Activated CNS antigen specific $CD4^+$ T cells are the only immune cell type that can induce EAE when transferred to immunocompetent recipient animals[121]. Although Th1 cells and Th17 cells can both induce EAE, activated myelin antigen specific Th1 cells mainly initiate spinal cord inflammation and activated myelin antigen specific cells; Th17 cells mainly promote inflammatory cell infiltration to the brainstem, cerebellum, and brain[122]. Before infiltrating the CNS, autoreactive T-helper cells are primed in the peripheral immune system. Encephalitogenic T cells are primed in skin draining lymph nodes after subcutaneous immunization of animals with myelin antigens or in mucosa associated lymphoid tissue in spontaneous animal models of EAE[123]. $CD8^+$ T cells are rarely found in experimental autoimmune encephalomyelitis lesions[53].

B cells are not needed for experimental autoimmune encephalomyelitis induced by MOG peptide. However, induction of this disorder with protein antigens (eg, human MOG protein) is dependent on B cell presence[124]. Therefore, the role of B cells in disease induction by MOG protein, in the animal model for EAE, is mainly associated with antigen processing and presentation[124].

## Objectives

The main aim of this study was to characterize the role of MAIT cells in the pathogenesis of MS.

## Experimental design

In the mouse studies we:

- Characterized the activation and migration of MAIT cells during EAE comparing mice immunized with $MOG_{35-55}$ and mice treated with complete Freund's adjuvant (CFA) only (control group)
- Characterized the effect of MAIT cell activation on EAE course and neuroinflammation using $MR1^{-/-}$ mice and $MR1^{+/-}$ and $MR1^{+/+}$ (MR1 sufficient) littermate controls

- Characterized the effect of different housing conditions on the phenotype we were observing by performing both co-housing experiments and experiments in which mice belonging to different groups were separately housed.

In the human study we:

- Quantified and characterized MAIT cells by flow cytometry in the peripheral blood (PB) and CSF of people with MS (pwMS) and healthy controls (HCs)
- Characterized cytokine production by MAIT cells obtained from pwMS and HCs after in vitro TCR stimulation with *E. coli*
- Characterized the effects of activated MAIT cells on in vitro $CD4^+$ T cell proliferation in pwMS and HCs

# Materials and methods

## Animals

Adult wild-type C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME), while $MR1^{-/-}$ mice were generated by Treiner and collaborators[86] and generously provided by S. Gilfillan (Washington University School of Medicine). The $MR1^{-/-}$ mice were originally generated on a mixed B6/129OlaHsd background. The mice were backcrossed to the C57BL/6N background (10 generations) and then the B6J background (10 generations). Male and female C57BL/6J and $MR1^{-/-}$ mice were housed in specific pathogen-free conditions in accordance with institutional and National Institutes of Health (NIH) guidelines. Animal protocols were approved by the Washington University Animal Studies Committee.

## EAE immunization

To induce EAE mice were immunized to induce EAE subcutaneously (sc) with 50 mg of myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ (MOG aa $_{35–55}$: MEV GWY RSP FSR VVH LYR NGK; Sigma Genosys), emulsified in incomplete Freund's adjuvant (IFA) containing 50 mg/mouse Mycobacterium tuberculosis (strain H37RA). Mice also received pertussis toxin (PT-300 ng/mouse; List Biological Laboratories) i.e. at the time of immunization and 48 h later[125]. In the control group mice received emulsified in incomplete Freund's adjuvant (IFA) containing 50 mg/mouse Mycobacterium tuberculosis (strain H37RA) without MOG and PBS or PT were

injected i.v. at the time of immunization and 48 h later. After immunization, mice were evaluated clinically in a blinded fashion using a disease severity scale that is scored on a 5-point scale: 0 = no disease; 1 = tail weakness; 2 = paraparesis; 3 = paraplegia; 4 = paraplegia with forelimb weakness or paralysis; 5 =moribund or dead animal, as described[126]. Littermate mice were randomized to different groups and co-housed from weaning up to the entire duration of the experiment to minimize littermate and cage effects. In selected experiments the effect of co-housing and separate housing was evaluated to describe the possible influence of the gut microbiome composition on disease course. All the described experiments were repeated three times for consistency.

### Isolation of splenocytes and lung mononuclear cells

Spleen of mice was mash through a 40 μm cell strainer using a syringe plunger. The strained was washed with 10 mL PBS and the suspended cells were centrifuged at 4 °C, 500G for 10°. The cell pellets were lysed using 5mL of ACK lysis buffer for 9 minutes, washed and resuspended in PBS 2% FBS.

Lungs were obtained from mice perfused with PBS and digested for 1h at 37 °C with collagenase II and DNase. Tissues were subsequently mashed through a 40 μm cell strainer and washed with PBS 2% FBS.

### Isolation of central nervous system mononuclear cells

Brain and spinal cord tissues from mice perfused with PBS were digested with collagenase IV (Sigma-Aldrich) for 30 min at 37 °C, resuspended in 37 % Percoll, and loaded between a 30 and 70 % Percoll gradient. After centrifugation at 2000g for 20 min, CNS mononuclear cells were retrieved from the 37/70 % Percoll interface as previously done[127].

### Isolation of small intestine lamina propria (SI-LP)

Small intestines of mice were dissociated by combining mechanical dissociation with enzymatic degradation using the lamina propria dissociation Kit (Miltenyi Biotec) per manufacturer's protocol. First intraepithelial lymphocytes (IELs) were dissociated from mucosa by shaking the tissue in a predigestion solution consisting of Hank's balanced salt solution (HBSS) with 10 mM Hepes, 5 mM EDTA, 5% fetal bovine serum, and 1 mM 1-4 dithiothreitol (DTT). Then, the small intestine lamina propria (SI-LP) tissue was enzymatically and mechanically dissociated into a

single-cell suspension using the gentleMACSTM Dissociators (Miltenyi Biotec). The resulting cells were further purified using a Percoll gradient (40%-70%).

## Subjects

PwMS and age-, gender- and BMI-matched HCs were recruited at the John L. Trotter Center, Washington University School of Medicine, St. Louis, (Missouri, USA) and at the Center for demyelinating and degenerative diseases at the IRCCS Policlinico Ca' Granda, Milan (Italy). PwMS met the 2017 revision of the McDonald criteria[115] for RRMS and they were all newly diagnosed and not treated with any DMTs. Exclusion criteria for both the MS and the control groups were cigarette smoking, steroid or antibiotic therapy in the last three months, active relapse (only for the MS group), other autoimmune or inflammatory diseases, immunosuppressive or immunomodulatory therapy, pregnancy. All subjects provided informed consent. This project was approved by the Institutional Review Board (IRB) at both sites.

## Flow cytometer analysis

In mouse studies, cells isolated from murine lungs, spleen, peripheric and mesenteric lymph nodes (MLN), CNS and SI LP were stained by flow cytometry. MR1-tetramer loaded with either 5-OP-RU or 6-FP (negative control) were kindly provided by the NIH tetramer core facility (Emory University). Surface staining including tetramers was performed by incubating the cells for 30 min at 4 °C. The following commercially available antibodies were used: anti-CD8 (53-6.7, BioLegend), anti-CD3 (17A2, BD Pharmingen), anti-CD19 (1D3, BD Pharmingen), anti-CD44 (IM7, BD Biosciences Pharmingen), anti-CD62L (MEL-14, Invitrogen), anti-CD45 (30-F11, BioLegend), anti-CD4 (GK1.5, BioLegend), anti-CD11c (N418, BioLegend), anti-Ly6G (RB6-BC5, Invitrogen), anti-CD11b (M1/70, BD Pharmingen), anti-Ly6C (HK1.4, eBioscience), anti-PD-1 (29F.1A12, BioLegend), anti-CD49d (R1-2, BioLegend), anti-F4/80 (BM8, BioLegend). Dead cells were excluded using the survival marker Zombie Aqua Dye (BioLegend). Staining for intracellular cytokines encompassed anti-mouse antibodies to IL-17A (TC11-18H10.1, BioLegend) and IFN-γ (XMG1.2, BioLegend). Isolated cells were incubated in 96 well-plates for 4.5 hours with 50 ng/ml of PMA and 750 ng/ml of ionomycin (both from Sigma) and monensin (eBioscience) at 37 °C. Surface staining was performed for 30 min at 4 °C. Subsequently, cells were fixed in 2% PFA for 10 min at room temperature (RT) and then permeabilized in saponin buffer for another 10 min

at RT. Cytokine staining was performed in permeabilization buffer for 30 min at 4 °C. Gating strategies are shown in **figure 4**.

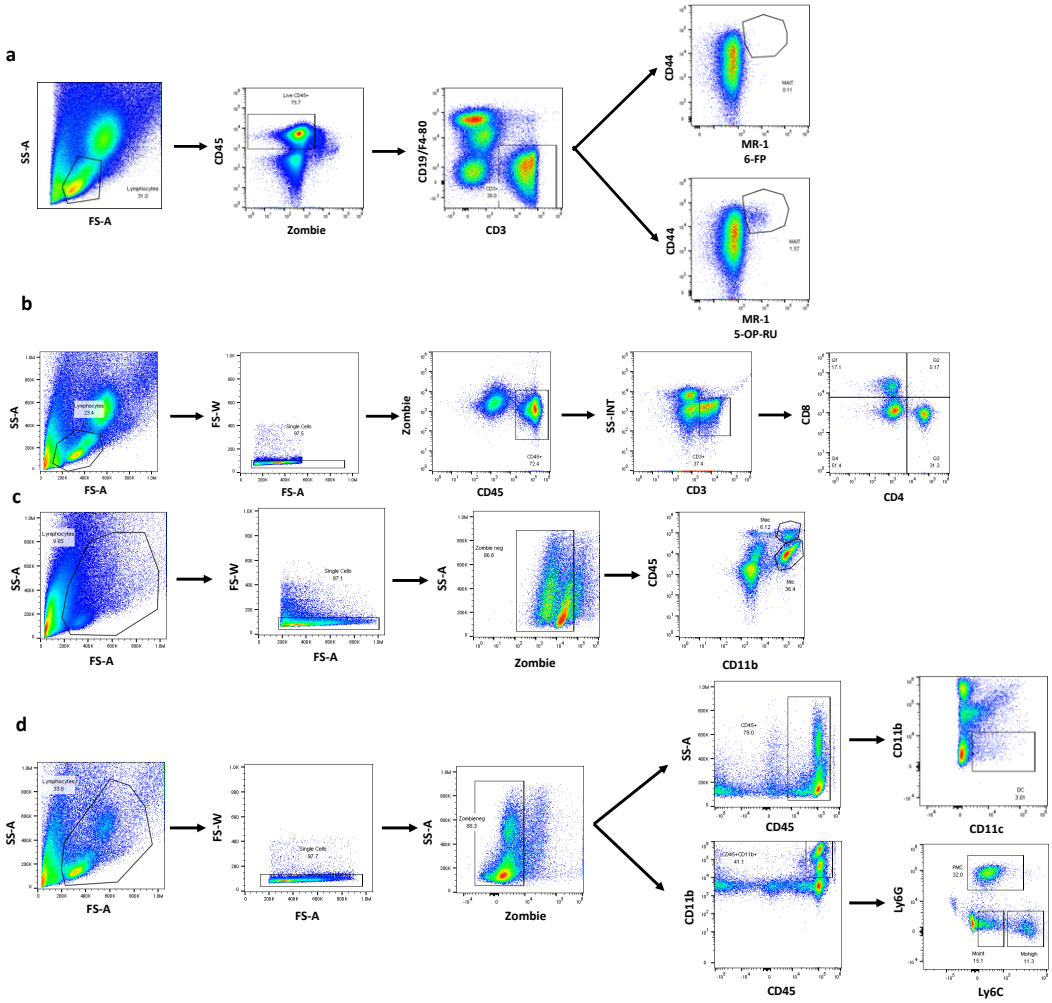


**Figure 4**. **Gating strategy for murine MAIT cells (a), T cells (b), brain (c) and peripheral organ (d) myeloid cell**. Mac=activated macrophages/microglia; Mic=resting microglia; DC=dendritic cells; PMN=polymorphonuclear cells; $Mo_{int}$=Ly6C$^{int}$ monocytes; $Mo_{high}$=Ly6C$^{high}$ monocytes.

In human studies, flow cytometry analyses were performed using anti-human antibodies to CD3 (sk7, BioLegend), CD4 (T4, Beckman Coulter), CD8 (SK1, BioLegend), V$\alpha$7.2 (3C10, BioLegend), CD196 (G034E3, BioLegend), CD197 (G043H7, BioLegend), CD183 (G025H7, BioLegend), CD161 (HP-3G10, BioLegend), PD-1 (EH12.2H7, BioLegend), CD45RA (HI100, BioLegend), starting from 100 ul of heparinized whole human blood which was incubated with the antibodies for 30 min at RT. Subsequently, samples were fixed and lysed with Step Fix/Lyse Solution (Affimetrix,

eBioscience) for 30 minutes at RT and washed following manufacturer's instructions. For intracellular staining peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient and incubated in an anti-CD28 (CD28.2, BioLegend, 2 μg/mL) coated 96-well plate with paraformaldehyde-fixed *E. coli* at a MOI of 6 for 24 hours at 37 °C. Monensin was added for the last 4 hours of incubation. Staining was performed as described above using antibodies anti-IL-17A (eBio64DEC17, Invitrogen), anti-IFN-γ (4S.B3, BioLegend), anti-IL-10 (JES3-9D7, BioLegend). In selected experiments anti-MR1 antibody (26.2; BioLegend) was added to the culture.

CSF samples were centrifuge at 400G for 10 minutes at 4 °C. Cells were stained for surface markers for 30 minutes at 4°C. **Figure 5** shows the gating strategy for the human staining.

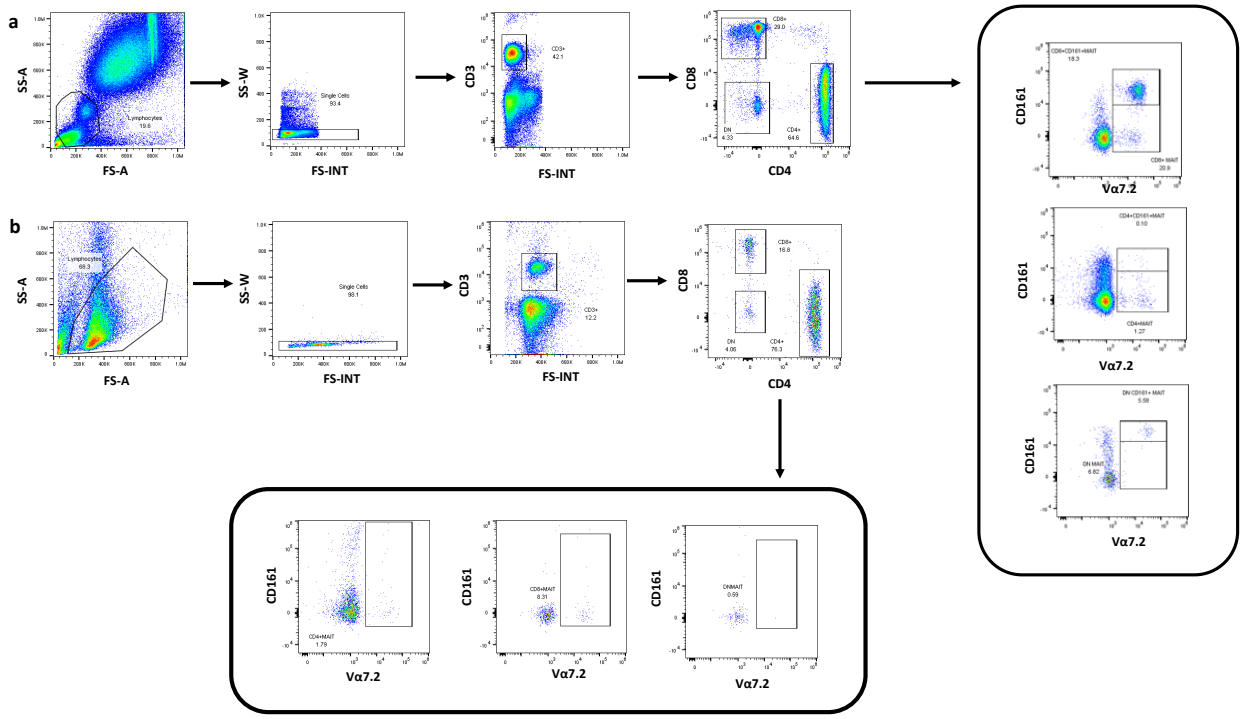


**Figure 5. Gating strategy for MAIT cells in human peripheral blood (a) and CSF (b) samples**.

To calculate absolute cell numbers, 100 microliters of 123count eBeads solution (Affimetrix, eBioscience) were added to each sample before flow cytometric analysis according to manufacturer's instructions. All data were analyzed using FlowJo™ ver. 10.7.2 (BD Bioscience).

## Proliferation assay

PBMCs were labeled with 1 μL CFSE (Invitrogen) and stimulated with paraformaldehyde-fixed *E. coli*, anti-CD3 (OKT3, BioLegend, 2 μg/mL) and anti-CD28 (CD28.2, BioLegend, 2 μg/mL). Cells

were incubated for 96h and CFSE dilution was measured by flow cytometry. In selected experiments $CD4^+$ T cells, monocytes and MAIT cells were isolated using micromagnetic anti-CD14, anti-CD4 and anti-FITC beads (Miltenyi Biotech, Berg. Gladbach, Germany). Briefly, MAIT cells were stained with FITC-conjugated anti-V$\alpha$7.2, washed, exposed to anti-FITC antibody-coated micromagnetic beads and passed through a magnetic isolation column. Monocytes and $CD4^+$ cells were separately incubated with anti-CD14 and anti-CD4 microbeads, respectively, washed and eluted through magnetic isolation columns. Cells eluted were approximately 90% pure. MAIT cells were stimulated with anti-CD28 (CD28.2, BioLegend, 2 μg/mL) and incubated overnight with *E. coli*-pulsed monocytes at a concentration of 1:1. Cells were then washed and co-cultured with CFSE-labelled $CD4^+$ T cells in an anti-CD3 and anti-CD28-coated 96 well plate, for 96 hours. CFSE dilution was then measured by flow cytometer.

## Blood sample preparation for metabolomics

Serum samples were vortexed with 80% chilled methanol aqueous in the ratio of 1:60 (v/v) to precipitate protein, following by centrifugation (14000 g, 4 ℃, 10 min). The supernatants were collected and dried under vacuum. The dried residues were reconstituted with 50% methanol aqueous and centrifuged (14000 g, 4 ℃, 15 min). We performed ultraperformance liquid chromatography-mass spectrometry (UPLC-MS) at the University of Massachusetts Amherst mass spectrometry facility to analyze serum for untargeted metabolomics. Equal volume of each serum sample was pooled to prepare the quality control (QC) sample. Five identical QC sample runs were conducted prior to running test samples, and one QC sample run was performed for every 8 sample runs throughout the experiment. Acquity UPLC HSS T3 column (2.1 mm×100 mm, 1.8 μm, Waters Co., MA, USA) was used to obtain the chromatographic separation by injecting 5 μL aliquots of each sample. The column was maintained at 40 ℃, and the flow rate was 0.5 mL/min. Solvent A was 95% water with 5% ACN and 0.1% formic acid and solvent B was 100% ACN with 0.1% formic acid. The gradient started at 2% of solvent B and linearly increased to 95% of solvent B at 8 min; held at 95% of solvent B for 2 min. The column was equilibrated at 2% of solvent B for 5 min before the next run. MS was conducted using the Thermo Fisher Orbitrap-Fusion in negative electrospray ionization mode at the detection range of 120-1000 m/z with 60000 full widths at half maximum resolution. The following conditions were used for MS: spray

voltage 3500 V, sheath gas flow rate 15 (arbitrary units), auxiliary gas flow rate 6 (arbitrary units), sweep gas flow rate 3 (arbitrary units), vaporizer temperature 275 ℃, ion transfer tube temperature 325 ℃.

## Statistical Analysis

### EAE experiments

EAE experiments were analyzed separately for disease incidence, mortality, day of onset and disease severity. Day of onset was considered as the first day in which neurological signs appear. Cumulative clinical score was the sum of all scores from onset to day 30 post-immunization (pi) or to the end of the experiment if the duration was < 30 days pi. Day of onset and cumulative clinical scores were summarized using the mean ± SEM. Maximum clinical score was calculated as the maximal score reached over the course of the disease. Differences in incidence between groups were analyzed by Fisher exact test. Disease clinical course was analyzed by two-way ANOVA in which the two factors were time and group. Other outcomes were analyzed using t-tests or the non-parametric Mann-Whitney test to compare differences between the two groups. An ANOVA or the equivalent non-parametric Kruskal-Wallis tests were used to analyze data with more than two groups. A P % 0.05 was considered significant. Analyses were performed using Prism v.6.03 software, GraphPad Prism 6.

### Human study

Demographic, anthropometric, and clinical characteristic of both pwMS and CTR in **table 2** and **3**. Differences in these characteristics between the two groups were compared using chi-square, t-tests, or the Mann-Whitney test, as appropriate.

For the metabolome data, the ion data set was subjected to peak detection, nonlinear alignment, and integration by XCMS (https://xcmsonline.scripps.edu). The data set in the mzXML data matrix converted by XCMS was normalized by sum of total of the observed peaks. The peaks with RSD > 20% in QC samples was excluded to guarantee the quality of data set, following by the univariate and multivariate analysis to differentiate the unbiased metabolites. We subjected resulting m/z values to the “MS peaks to pathways” analysis in Metaboanalyst (https://www.metaboanalyst.ca/) to analyze pathways and identify metabolites with a maximum error of 5 ppm using KEGG and Metlin databases. Welch’s t-test was used to determine significant

changes between the control and MS groups. P values were further adjusted by FDR approach. All p-values were two-tailed.

# Results

## MAIT cells are enriched in the lungs and in the brain during EAE.

C57BL/6 mice were immunized with $MOG_{35-55}$ as previously described[125] or with CFA only + PBS (control group). Mice were sacrificed at 7 days post-immunization (dpi - pre-clinical phase) and at disease peak (14-15 dpi). Peripheric lymph nodes (PLN), small intestine lamina propria (SI-LP), spleen, lungs and CNS were collected (**Figure 6**).

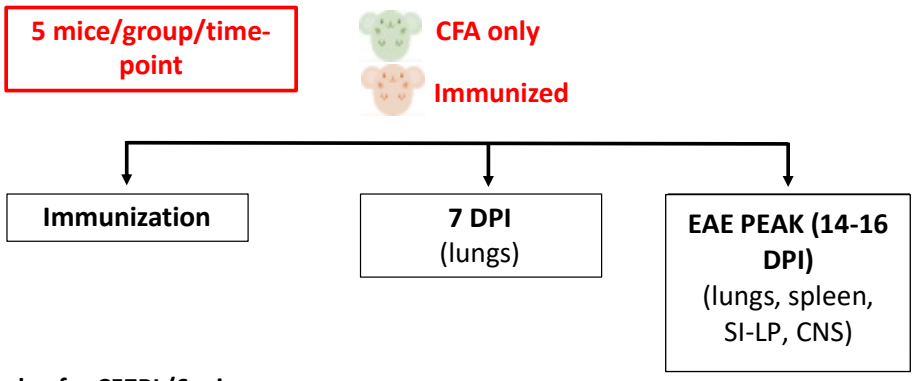


**Figure 6. Study plan for C57BL/6 mice.**

MAIT cells were quantified as $CD45^+Zombie^-CD3^+CD19^-F4/80^-CD44^+MR1^+$ cells. PD1 and VLA-4 expression was quantified as mean fluorescent intensity (MFI). At 7 dpi, MAIT cells were enriched in the lungs of $MOG_{35-55}$ compared to CFA-only immunized mice, in terms of both percentages and absolute numbers (**Figure 7a-b**). Furthermore, we observed an upregulation of the inhibitory molecule PD1 and the surface molecule VLA-4 by MAIT cells obtained from $MOG_{35-55}$ immunized mice compared to CFA control group (**Figure 7c**). No differences were observed in the lungs at 14 dpi.

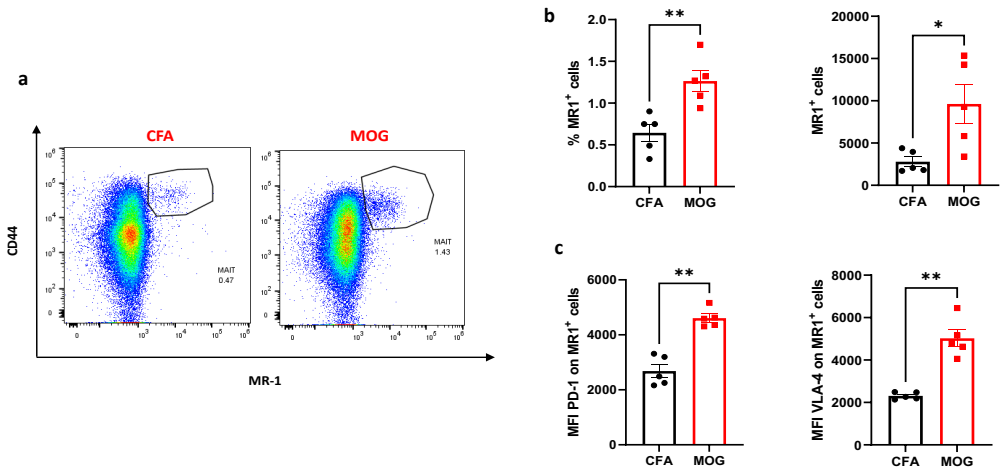


**Figure 7. Analysis of MAIT cells in the lungs of CFA versus $MOG_{35-55}$-immunized mice at 7 dpi.** Exemplificative flow-cytometry plot of MAIT cells (a). MAIT cells were higher in $MOG_{35-55}$-immunized mice compared to the CFA control groups both in percentages and absolute numbers (b). MAIT cells obtained from $MOG_{35-55}$-immunized upregulated PD1 and VLA-4 (c). **p<0.05; **p<0.01.*

As expected, no MAIT cells were identifiable in the peripheric lymph nodes obtained from mice of both groups (data not shown).

In the spleen, we described a trend towards an increase in percentages of MAIT cells in $MOG_{35-55}$ immunized mice, but no differences in absolute numbers or in PD1 expression during EAE peak (**Figure 8**).

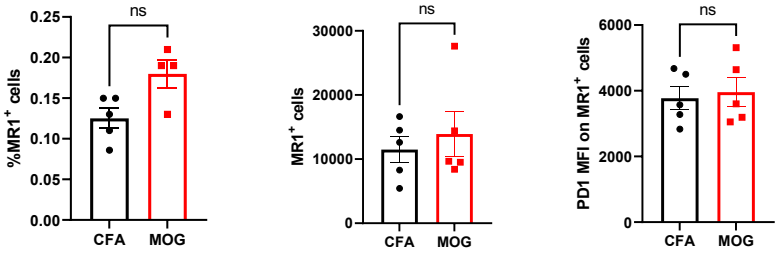


**Figure 8. MAIT cells in the spleen of CFA versus $MOG_{35-55}$-immunized mice at 14 dpi**. No differences in absolute numbers or PD-1 expression were observed in the spleen of immunized mice compared to the CFA group. A trend toward a higher percentage of MAIT cells in immunized mice was described at 7 dpi.

At 14 dpi, MAIT cells were decreased in the SI-LP in both percentages and absolute numbers, with results close to statistical significance and they tended to upregulate the surface molecule PD1 (**Figure 9**).

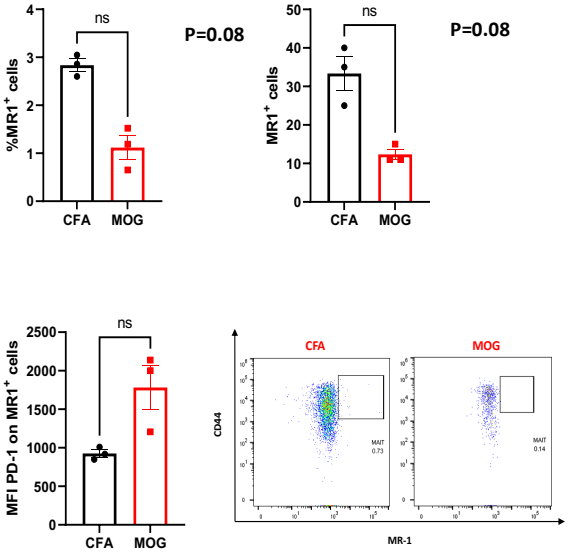


**Figure 9. MAIT cells obtained from the small intestine lamina propria at 14 dpi.** Percentages and absolute numbers of MAIT cells were lower and PD-1 expression was higher in $MOG_{35-55}$-immunized mice compared to CFA control group.

In the CNS, we observed an increase in the percentages of MAIT cells at 14 dpi, following the development of clinical symptoms (**Figure 10**).

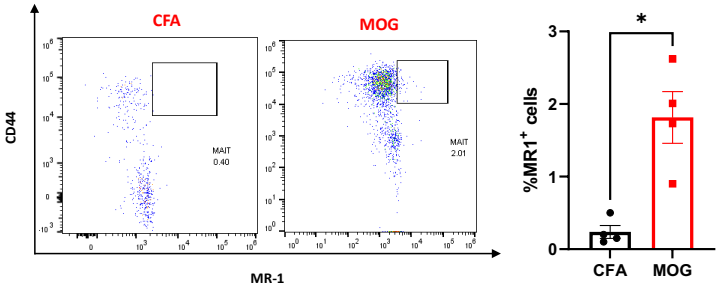


**Figure 10**. **MAIT cells obtained from the CNS at 14 dpi**. MAIT cells were higher in $MOG_{35-55}$- versus CFA-immunized mice. **p<0.05.*

To exclude that the differences we observed were caused by the pertussis toxin (PT) injection, we compared two groups of mice, one immunized with CFA+PBS and the other with CFA+PT. As shown in **Figure 11** no differences in MAIT cell number or PD1 expression were observed in the lungs or in the CNS, at 7 and 14 dpi respectively.

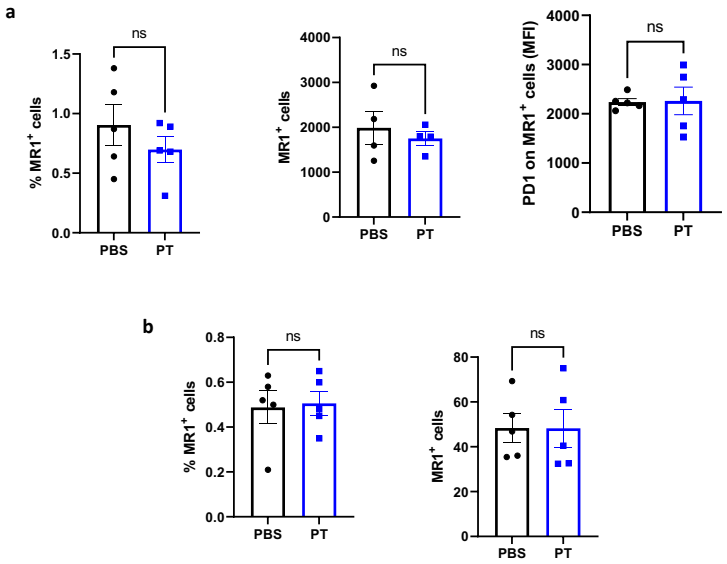


**Figure 11. MAIT cells in the lungs and CNS of mice immunized with CFA+PBS (a) compared to mice immunized with CFA+PT (b)**. No differences were observed in terms of percentages, absolute numbers or PD1 expression.

## MAIT cells obtained from the lungs of immunized mice have a MAIT1 phenotype

Cells were isolated from the lungs of both EAE and CFA-treated mice and stimulated in vitro with PMA, ionomycin and monensin. At 7 dpi, MAIT cells obtained from the lungs of immunized mice showed a predominant production of IFN-γ after in vitro stimulation (MAIT1). On the contrary MAIT cells obtained from mice treated only with CFA showed a predominant IL-17A production after in vitro stimulation (MAIT17) (**Figure 12**).

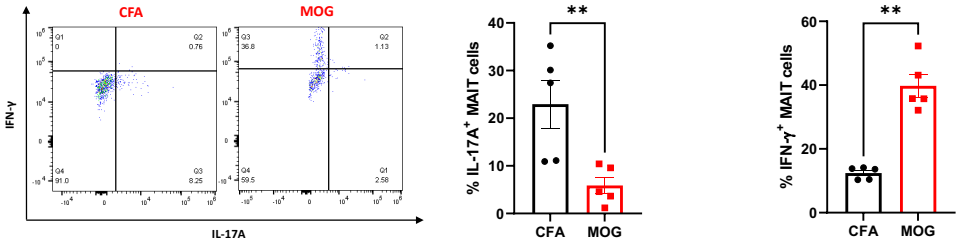


**Figure 12**. **IL-17A and IFN-γ production by MAIT cells.** MAIT cells obtained from $MOG_{35-55}$-immunized mice showed a clear MAIT1 phenotype. ***p<0.01.*

## MR1$^{-/-}$ mice have a more severe disease course

To inquire the effect of MAIT cell activation on EAE we used MR1$^{-/-}$ mice, lacking completely MAIT cells[86]. The EAE clinical course of MR1$^{-/-}$ was compared to MR1$^{-/+}$ or MR1$^{+/+}$ (MR1 sufficient) littermate controls (**Figure 13**). Mice were sacrificed at 7 dpi, at disease peak (14-16 dpi) and during the chronic phase of EAE and lungs, CNS, mesenteric lymph-nodes (MLN) and SI-LP were obtained.

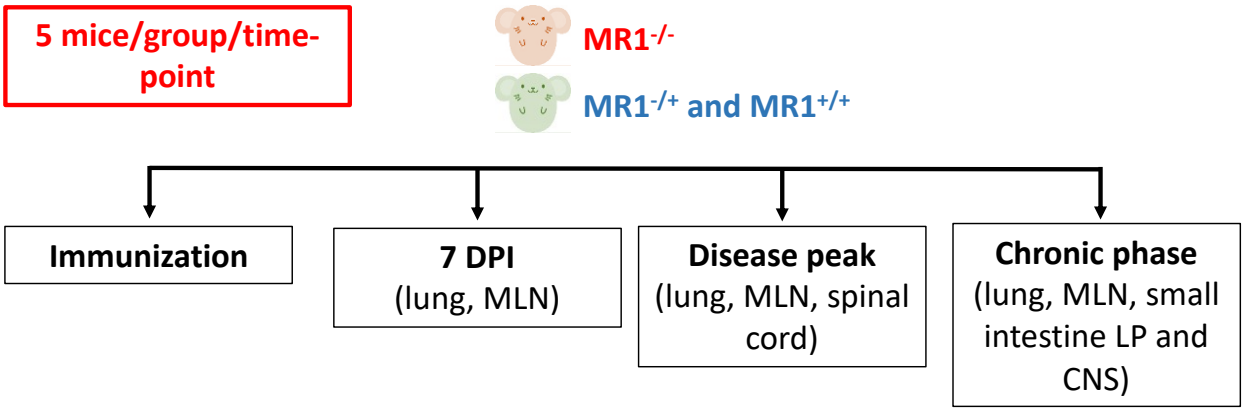


**Figure 13. Study plan for MR1$^{-/-}$ mice.**

MR1$^{-/-}$ showed a more severe disease course compared to heterozygous or wild-type littermates (**Figure 14**). The difference was statistically significant. No differences in term of disease onset and mean disease score were observed.

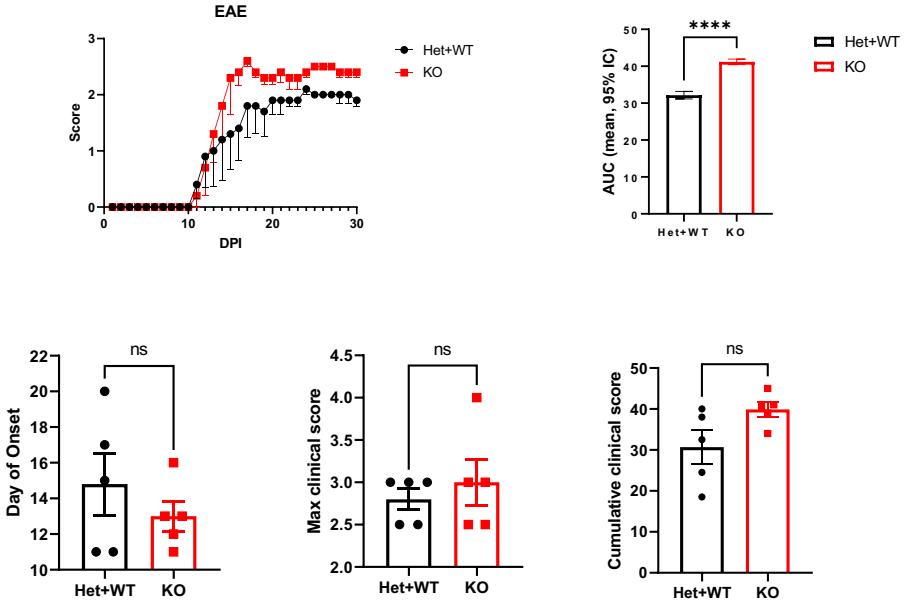


**Figure 14**. **Clinical course of MR$^{-/-}$ mice.** MR1$^{-/-}$ mice had a more severe clinical course with a higher cumulative score compared to heterozygous or wild-type littermates. *****p<0.001.*

## MR1$^{-/-}$ mice show higher numbers of CD4$^{+}$ infiltrating lymphocytes and lower numbers of homeostatic microglia cells in the CNS.

Lymphocytes and microglia cells were quantified as CD45$^{+}$Zombie$^{-}$CD3$^{-}$CD19$^{+}$ (B cells), CD45$^{+}$Zombie$^{-}$CD3$^{+}$CD19$^{-}$CD4$^{+}$ or CD8$^{+}$ (T cells), Zombie$^{-}$CD45$^{high}$CD11b$^{high}$ (activated, microglia/macrophages), Zombie$^{-}$CD45$^{low}$CD11b$^{high}$ (resting microglia). The CNS was obtained from MR1$^{-/-}$ mice and littermate controls during the chronic phase of the disease. There were not differences in terms of total number of infiltrating lymphocytes or total myeloid cells (data not shown). However, MR1$^{-/-}$ mice showed higher percentages and absolute numbers of CD4$^{+}$ lymphocytes and lower percentages of homeostatic microglia, defined as CD45$^{low}$CD11b$^{high}$ cells (**Figure 15**). To further confirm the higher pro-inflammatory state of the CNS of MR1$^{-/-}$ mice, CD4$^{+}$ T cells had a higher expression of the effector molecule CD44, and microglia cells had a lower expression of the homeostatic molecule P2RY12 (**Figure 15**) compared to both heterozygous and wild-type littermates.

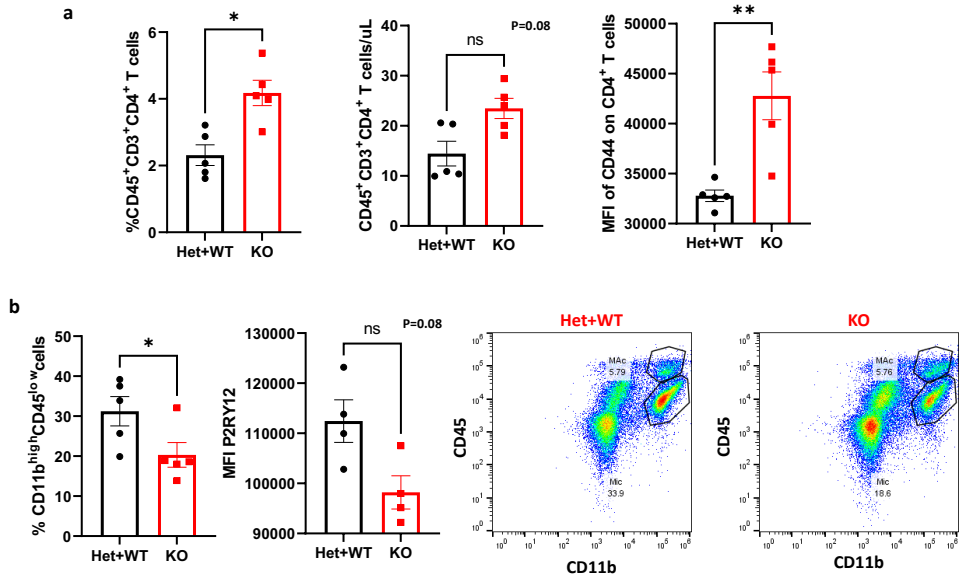


**Figure 15. Microglia and infiltrating lymphocytes in MR1$^{-/-}$ mice.** CD4$^{+}$ T cells are higher and express higher levels of CD44 in MR1$^{-/-}$ mice compared to littermate controls (a). Homeostatic microglia are lower and express lower levels of P2RY12 (b).

No differences in other cell subset were observed (data not shown).

## MR1$^{-/-}$ mice have lower numbers of pro-inflammatory monocytes in the lamina propria and higher numbers in the lungs during EAE

Lymphocytes and myeloid cells were quantified as CD45$^{+}$Zombie$^{-}$CD3$^{-}$CD19$^{+}$ (B cells), CD45$^{+}$Zombie$^{-}$CD3$^{+}$CD19$^{-}$CD4$^{+}$ or CD8$^{+}$ (T cells), CD45$^{+}$Zombie$^{-}$CD11b$^{+}$Ly6G$^{+}$Ly6C$^{+}$ (polymorphonuclear leukocytes – PMN), CD45$^{+}$Zombie$^{-}$CD11b$^{+}$Ly6G$^{-}$Ly6C$^{int/high}$ (monocytes), CD45$^{+}$Zombie$^{-}$CD11b$^{-}$CD11c$^{+}$ (dendritic cells – DC). Percentages and absolute numbers of Ly6C$^{high}$ proinflammatory monocytes tended to be higher in the lungs (**Figure 16a**) and lower in both the MLN and the SI-LP of MR1$^{-/-}$ mice during EAE (**Figure 16b-c**).

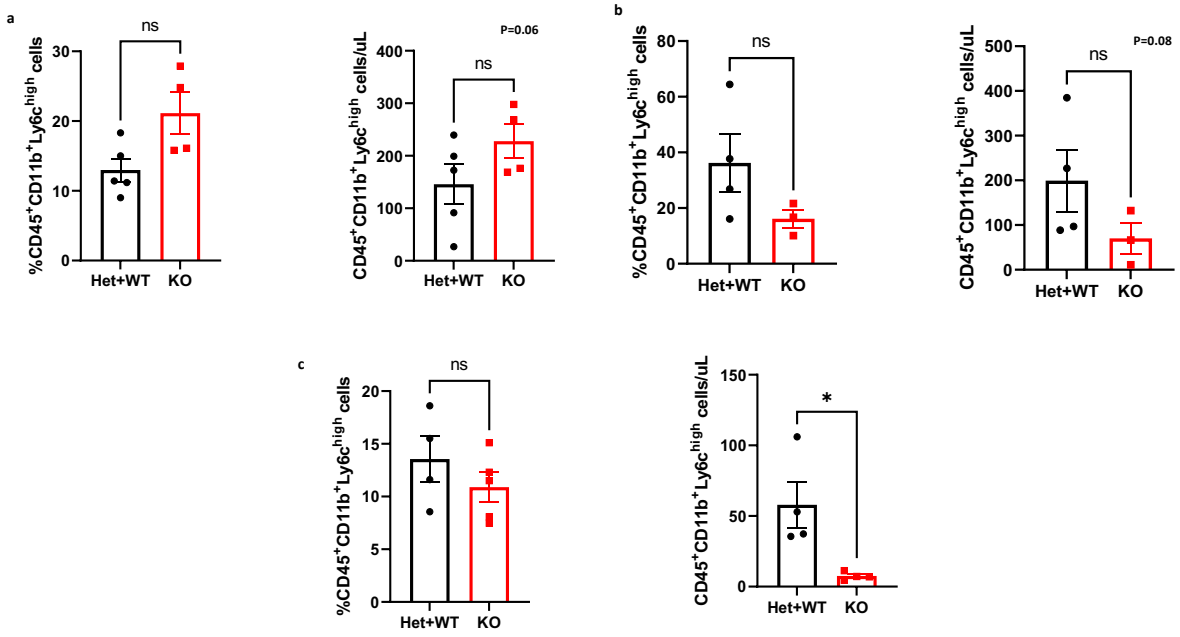


**Figure 16. Ly6c$^{high}$ monocytes in the lungs, MLN, and SI-LP**. Pro-inflammatory monocytes were higher in the lungs (a) and lower in the lamina propria (b) and MLN (c) of MR1$^{-/-}$ mice.

Furthermore, we described lower percentages of CD4$^{+}$ effector cells in the mesenteric lymph-nodes of MR1$^{-/-}$ mice compared to MR1 sufficient littermates. CD4$^{+}$ T cells obtained from MR1$^{-/-}$ mice showed also lower expression of CD44 compared to heterozygous or wild-type mice (**Figure 17**)

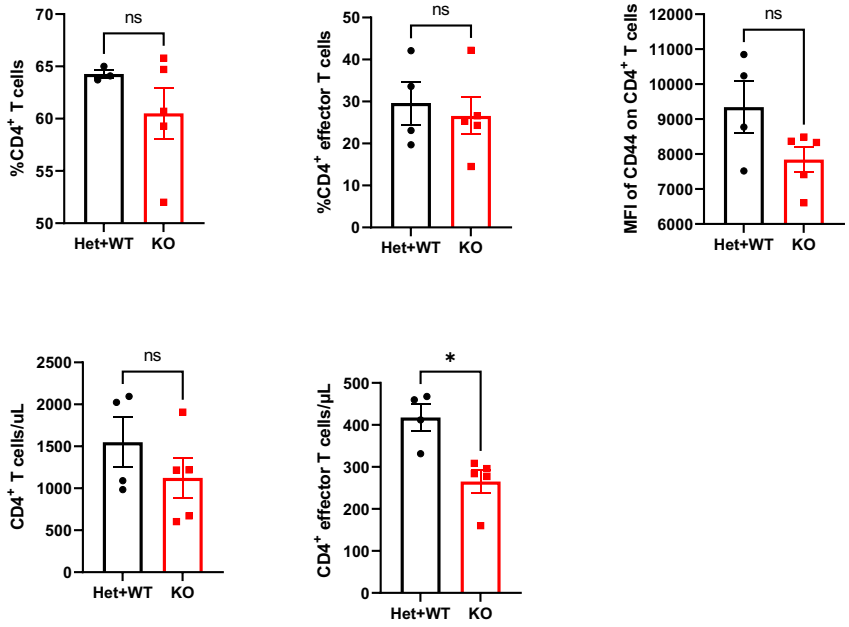


**Figure 17. CD4$^+$ T cells in MLN at disease peak**. CD4$^+$ showed lower CD44 expression and CD4+ effector cells were lower in MLN of MR1$^{-/-}$ mice compared to MR1 sufficient controls.

## Before the development of clinical EAE MR1$^{-/-}$ mice showed higher inflammation in peripheric lymphoid organs and in the lungs

We characterize the immunophenotype of MR1$^{-/-}$ mice and MR1 sufficient controls at 7 dpi, before the development of clinical EAE, in the peripheric lymph nodes and lungs. MR1$^{-/-}$ mice showed higher numbers of dendritic cells, monocytes and PMN in the lungs and higher numbers of both CD4$^+$ and CD19$^+$ cells in the MLN. Finally, MR1$^{-/-}$ mice tended to have higher numbers of monocytes also in the inguinal and axillary lymph-nodes, confirming a higher inflammatory response to immunization compared to MR1 sufficient littermates (**Figure 18**).

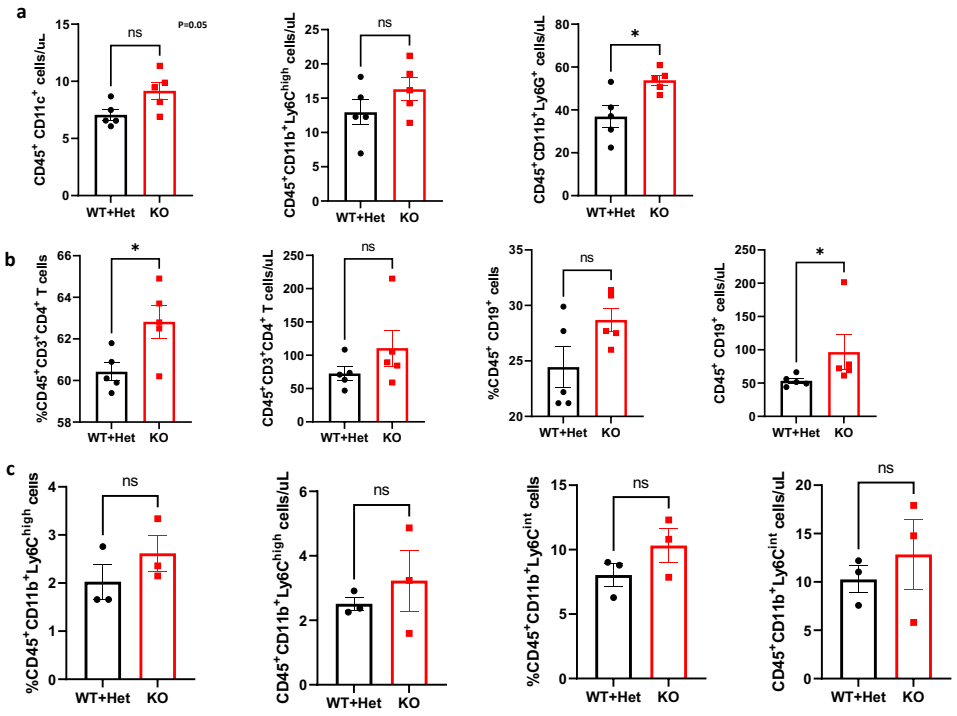


**Figure 18. PLN, M LN, and lung immunophenotype in MR1$^{-/-}$ and MR1 sufficient mice at 7 dpi.** MR1$^{-/-}$ showed higher numbers of DC, monocytes and PMN in the lungs (a), higher numbers of CD4$^{+}$ and CD19$^{+}$ cells in the MLN (b), and higher percentages of myeloid cells in the PLN (c) at 7 dpi compared to MR1 sufficient littermates. * $p < 0.05$.

## The immunomodulatory role of MAIT cells during EAE is influenced by the housing conditions.

MAIT cell maturation and function is influenced by the gut microbiome. In mice, their definite phenotype is determined by the gut microbiome composition during the first three weeks of life, with small changes in their frequency and activation profile driven by the micro-organisms at the site of inflammation still possible, even during adulthood[95]. Co-housing mice belonging to different groups is considered a proxy of a fecal matter transplantation procedure[128]. To inquire a possible influence of the gut microbiome in modulating the role of MAIT cells during neuroinflammation we repeated our experiment in different housing conditions. We compared the clinical course of MR1$^{-/-}$ to MR1 sufficient (MR1$^{-/-}$ and MR1$^{+/-}$) littermates either co-housed or housed separately since weaning. When housed separately MR1$^{-/-}$ developed a less severe clinical course compared to MR1 sufficient mice (**Figure 19**). These observations led to hypothesize an

important role of the gut microbiome in shaping MAIT cell responses during the development on neuroinflammation.

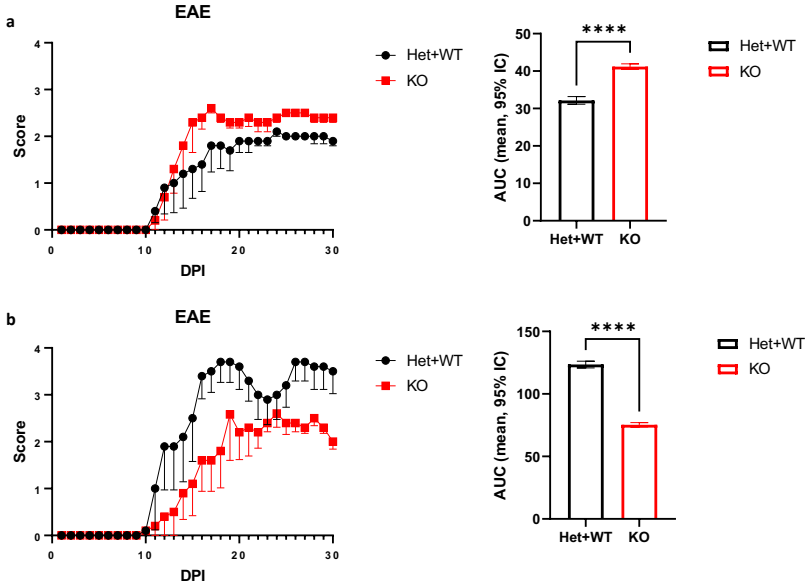


**Figure 19. EAE clinical course in MR1$^{-/-}$ and MR1 sufficient mice.** The clinical course of MR1$^{-/-}$ mice is dependent on housing conditions at immunization. They developed a more severe clinical course when co-housed (a), a less severe clinical course when house separately compared to littermate controls (b)

## CD8$^{+}$CD161$^{high}$ MAIT cells are lower in peripheral blood samples obtained from pwMS compared to controls

We obtained peripheral blood samples from a total of 32 pwMS and 24 healthy controls (CTR). **Table 2** summarizes the characteristics of the study cohorts. MAIT cells were quantified and characterized both as total CD45$^{+}$CD3$^{+}$CD4$^{+}$V$\alpha$7.2$^{+}$, CD45$^{+}$CD3$^{+}$CD8$^{+}$V$\alpha$7.2$^{+}$, CD45$^{+}$CD3$^{+}$CD4$^{-}$CD8$^{-}$V$\alpha$7.2$^{+}$ cells and as the subpopulation of CD45$^{+}$CD3$^{+}$CD4$^{+}$CD161$^{high}$V$\alpha$7.2$^{+}$, CD45$^{+}$CD3$^{+}$CD8$^{+}$CD161$^{high}$V$\alpha$7.2$^{+}$ and CD45$^{+}$CD3$^{+}$CD4$^{-}$CD8$^{-}$CD161$^{high}$V$\alpha$7.2$^{+}$ cells. PD-1, CCR6 and CXCR3 expression was quantified as MFI. Naïve, central memory, effector and effector memory subpopulation were quantified based on the expression of the chemokine CCR7 and of the surface protein CD45RA. Th1-, Th2- and Th17-like phenotypes were further quantified based on the differential expression of the surface molecules CCR6 and CXCR3.

| PERIPHERAL BLOOD SAMPLES | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | WashU | | | Unimi | | | P value | |
| | MS | CTR | P value | MS | CTR | P value | MS | CTR |
| Number (n) | 15 | 12 | N/A | 17 | 12 | N/A | N/A | |
| Sex (F:M) | 9:6 | 8:4 | 0.12 | 13:4 | 10:2 | 0.65 | 0.32 | 0.34 |
| Age (mean±SD) | 37.47±9.47 | 34.58±8.74 | 0.42 | 35.8±10.28 | 34±7.24 | 0.58 | 0.64 | 0.86 |
| BMI (mean±SD) | 25.52±5.31 | 24.24±4.24 | 0.63 | 23.4±4.56 | 22.58±4.34 | 0.62 | 0.23 | 0.35 |
| OCB + (n, %) | 13 (86.6%) | N/A | N/A | 14 (82.35%) | N/A | N/A | 0.87 | |

**Table 2. Characteristics of the two cohort of subjects recruited at Washington University in St. Louis and IRCCS Policlinico Ca' Granda for peripheral blood samples.** OCB=oligoclonal bands.

Total CD8⁺ MAIT cells and CD8⁺CD161$^{high}$ MAIT cells were lower in both percentages and absolute numbers, in peripheral blood samples obtained from pwMS compared to CTR (**Figure 20**).

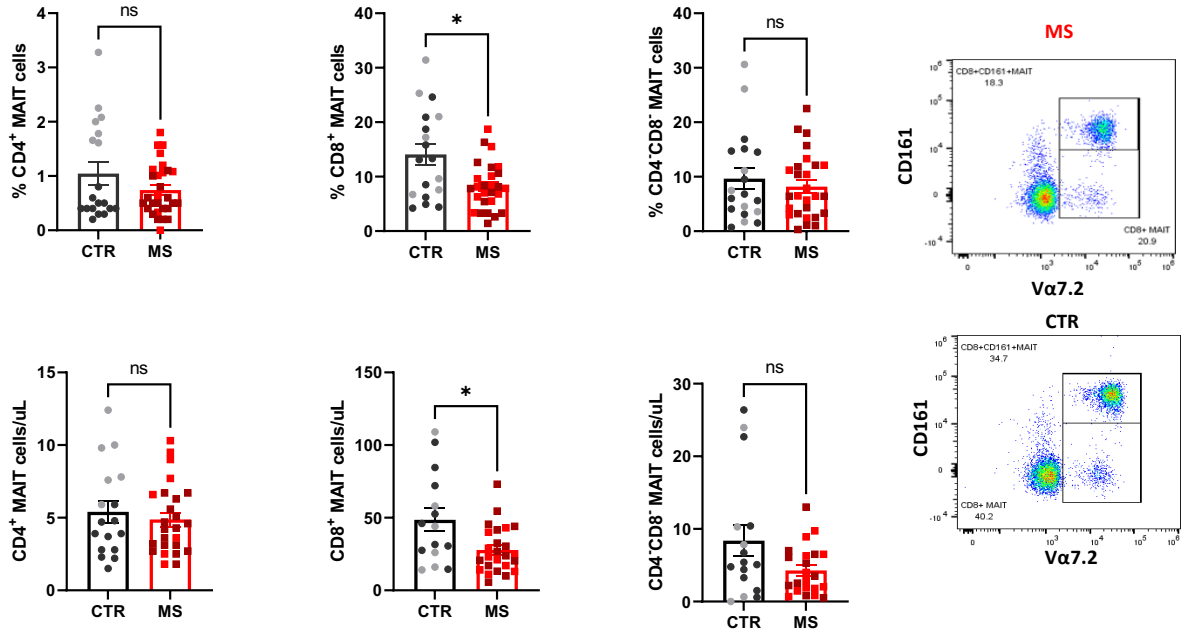


**Figure 20. Percentages and absolute numbers of CD8⁺ MAIT cells.** MAIT cells were lower in pwMS compared to HCs. CTR=controls. ** p < 0.05*

Furthermore, CD8⁺ MAIT cells showed lower expression of the inhibitory molecule PD1 in pwMS compared to CTR (**Figure 21**). The analysis of MAIT cell maturation state using CD45RA and CCR7 didn't show any difference between pwMS and CTR. Similarly, there were no differences in Th-1-, Th-2- or Th-17-like subtype representation between the cohorts (data not shown).

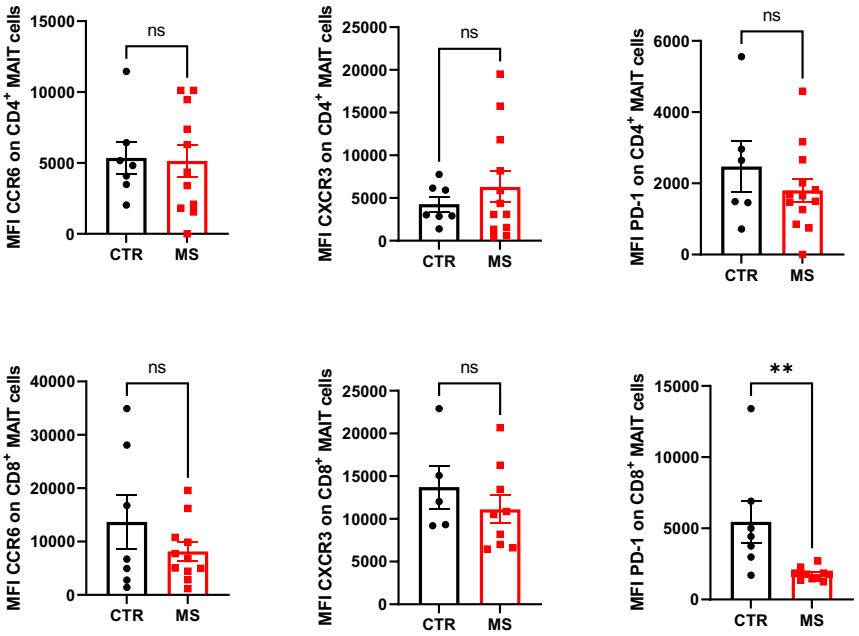


**Figure 21**. **PD-1 expression on CD8⁺ MAIT cells.** MAIT cells obtained from pwMS showed lower expression of the inhibitory molecule PD-1. *** p < 0.01*

## CD8⁺ and CD4/CD8 double negative MAIT cells are lower in CSF samples obtained from pwMS compared to controls

During acute and chronic inflammation MAIT cells migrate to the site of inflammation and a decrease in their circulating number has been described. To inquire whether MAIT cells migrate inside the CNS of pwMS, CSF samples were obtained from 30 pwMS and 8 subjects who underwent a lumbar puncture in the suspect of other neurological diseases, not confirmed at follow-up. **Table 3** summarizes the characteristics of the study cohorts.

| CSF SAMPLES | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | WashU | | | Unimi | | | P value | |
| | MS | CTR | P value | MS | CTR | P value | MS | CTR |
| Number (n) | 13 | 3 | N/A | 17 | 5 | N/A | N/A | |
| Sex (F:M) | 9:4 | 2:1 | 0.93 | 13:4 | 3:2 | 0.46 | 0.65 | 0.85 |
| Age (mean±SD) | 35.69±8.51 | 37.5±9.21 | 0.82 | 35.8±10.28 | 42.5±7.77 | 0.15 | 0.97 | 0.47 |
| BMI (mean±SD) | 24.83±6.41 | 23.52±6.13 | 0.72 | 23.4±4.56 | 22.34±2.15 | 0.48 | 0.50 | 0.77 |
| OCB + (n, %) | 13 (100%) | N/A | N/A | 14 (82.35%) | N/A | N/A | 0.42 | |

**Table 3**. **Characteristics of the two cohort of subjects recruited at Washington University in St. Louis and IRCCS Policlinico Ca' Granda for CSF samples**. OCB=oligoclonal bands.

Compared to peripheral blood samples, CSF samples showed a lower number of CD161$^{high}$ MAIT cells (**Figure 22**). However, CSF samples obtained from pwMS showed lower percentages of CD8⁺

and double negative MAIT cells compared to CSF samples obtained from HCs (**Figure 22a**). Furthermore, despite the low number of samples included in the analysis, the expression of the inhibitory molecule PD-1, was significantly lower in pwMS compared to CTR (**Figure 22b**).

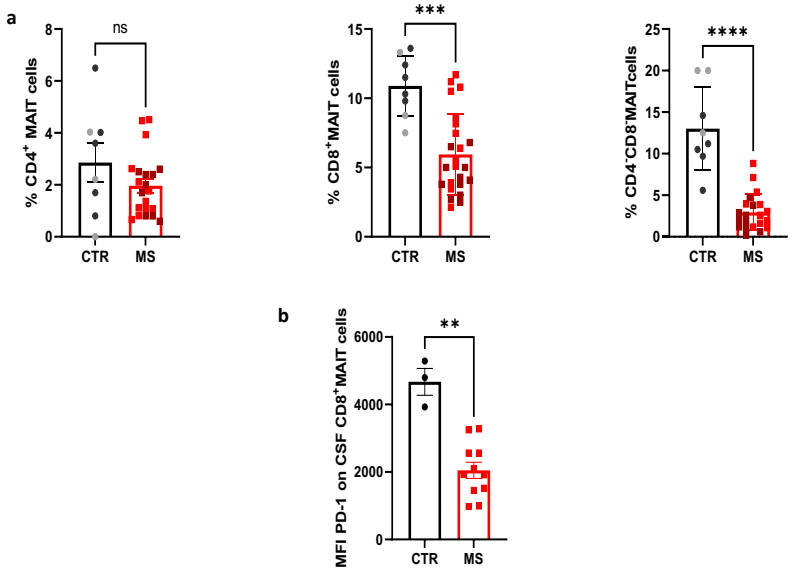


**Figure 22**. **Percentages and PD-1 expression of MAIT cells in the CSF**. MAIT cells were lower in CSF samples obtained from pwMS compared to CTR (a), with lower expression of the inhibitory molecule PD-1 (b). *** p<0.01; *** p<0.001; ****p<0.0001*

Finally, we described a significant correlation between blood and CSF MAIT cell in pwMS (**Figure 23**), further supporting our results and the notion that the lower number of MAIT cells described in pwMS is not due to their migration inside the CNS.

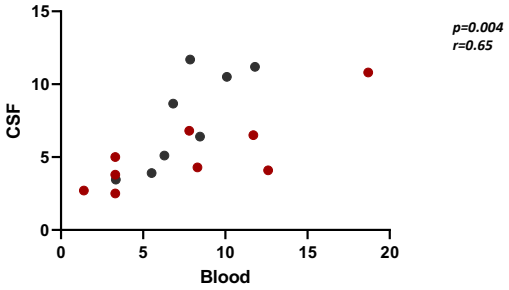


**Figure 23**. **Correlation between peripheral blood and CSF MAIT cells in pwMS.** There was a strong positive correlation between the percentages of MAIT cells in the PB and in the CSF of pwMS.

## CD8⁺ MAIT cells obtained from pwMS show defective production of IFN-γ after stimulation with *E. coli*

PBMCs were stimulated with anti-CD28 and paraformaldehyde-fixed E. Coli for 24 hours. Monensin was added for the last 4 hours of stimulation. CD8⁺ MAIT cells obtained from pwMS produced significant lower levels of IFN-γ compared to CD8⁺ MAIT cells obtained from HCs (**Figure 24)**.

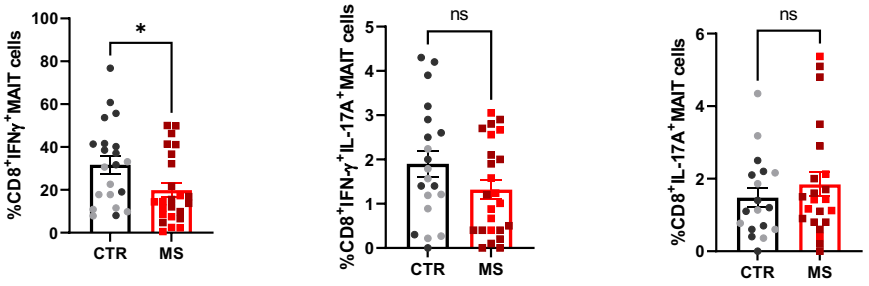


**Figure 24. Cytokine production by CD8⁺ MAIT cells after stimulation with E. Coli.** CD8⁺ MAIT cells obtained from pwMS showed significant lower production of IFN-γ compared to CD8⁺ MAIT cells obtained from HCs.

## MAIT cells activation suppress CD4⁺ T cells proliferation in vitro

PBMCs were stimulated with anti-CD3 and anti-CD28 with or without paraformaldehyde-fixed E. Coli for 96 hours. CD4⁺ T cells proliferated strongly without E. Coli in samples obtained from both pwMS and HCs. After activation of MAIT cells by E. Coli, CD4⁺ T cells proliferation was inhibited efficiently in samples obtained from HCs, but not in samples obtained from pwMS (**Figure 25**).

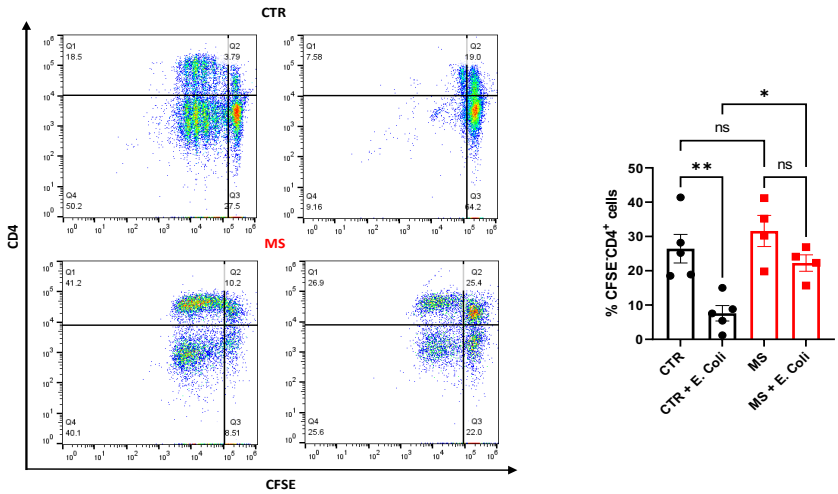


**Figure 25. Proliferation assay on CD4⁺ cells after activation of MAIT cells.** CD4⁺ T cells proliferation was inhibited after activation of MAIT cells in HCs but not in pwMS. CTR=controls. ** p<0.05; **p<0.01*.

## PwMS show an increased abundancy of riboflavin-producing micro-organisms in their gut microbiome and an upregulation of riboflavin metabolic pathway in the serum

Following our observations in the mouse model of the disease, we inquired if also in pwMS the gut microbiome could influence the role of MAIT cells. A subgroup of pwMS enrolled in this study was also recruited for another study on the characterization of the gut microbiome. We described an increased abundancy of the fungi *Saccharomyces cerevisiae* and *Aspergillus fumigatus* in the gut mycobiome of this cohort of pwMS compared to HCs[129]. Since both genera are known producer of riboflavin, we re-analyze the metabolome data available focusing on the riboflavin metabolic pathway. As expected, we confirmed an increase in the riboflavin metabolic pathway in pwMS compared to HCs, despite no difference in riboflavin intake was evident at the food diary collection (**Figure 26**).

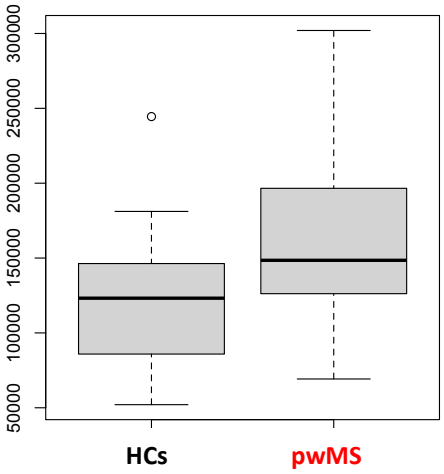


**Figure 26**. **Riboflavin metabolic pathway in HCs and pwMS**. PwMS showed an upregulation of riboflavin and its metabolites in serum samples.

## Conclusions

In this study we described an immunoregulatory role of MAIT cells during EAE, influenced by the environment. In wild-type mice we observed an enrichment in MAIT cells in the lungs at 7 dpi, before the development of clinical EAE. MAIT cells isolated from the lungs of immunized mice showed an upregulation of the molecules VLA-4 and PD1. The very late antigen 4 (VLA-4) is the ligand for the vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells, and one of the surface molecules responsible for the successful extravasation of lymphocytes inside the CNS[130]. Our observation of an enrichment in VLA-4$^+$ MAIT cells in the lungs of immunized mice is in line with previous studies showing how T cells become licensed in the lungs to enter the CNS during EAE[131]. Accordingly, we observed a subsequent enrichment in MAIT cells in the CNS during clinical EAE. PD1 is an activation marker, that can be upregulated early during the immune response to secure a subsequent negative feedback and prevent overactivation[132]. MAIT cells upregulate PD1 expression in response to direct TCR engagement, with the highest levels described in colon-resident MAIT cells[97]. Despite our observation could be explained by the exposition of MAIT cells to a pro-inflammatory environment during immunization, it's also possible that changes in the microbiome following immunization are responsible for the upregulation of PD1. We observed a decrease in MAIT cell frequency in the SI-LP of immunized mice during clinical EAE. MAIT cells can be activated by the gut microbiome and migrate from the intestinal mucosa to the lungs before entering the CNS. In line with this hypothesis, MAIT cells obtained from immunized mice showed a MAIT1 phenotype, with a predominant production of IFN-γ, which is usually a consequence of direct TCR stimulation by riboflavin-metabolites. On the contrary, MAIT cells obtained from CFA control mice showed a MAIT17 phenotype. Moreover, the production of both IL-17 and IFN-γ by MAIT cells has been linked to their pro-inflammatory role in the activation of monocytes and in the polarization of other T cells (Th1 and Th17)[97]. However, Ni and colleagues hypothesized a stabilizing effect of IFN-γ on the BBB during the development of EAE[133] and absence of IFN-γ has been associated with worse disease pathology during the development of EAE[134].

To further understand the role of MAIT cells during neuroinflammation we used MR1$^{-/-}$ mice, lacking completely MAIT cells. MR1$^{-/-}$ developed a more sever clinical course and higher numbers

of pro-inflammatory myeloid and lymphoid cells in peripheral lymph nodes, lungs, and CNS during clinical EAE, compared to littermate MR1 sufficient mice. Our data supported a regulatory role of MAIT cells during clinical EAE. However, we couldn't ignore that MAIT cell function is linked to the microbiome composition and that we eliminated this influencing factor by using co-housed littermates for our experiments. To overcome this limitation, we repeated the previous experiments comparing the clinical course between $MR1^{-/-}$ mice and co-housed or separately housed MR1 sufficient littermates. $MR1^{-/-}$ mice resulted protected when separately housed but showed a worse disease course when co-housed. These results further confirm that the microbiome could deeply influence MAIT cell function during neuroinflammation and that, depending on the environmental stimuli they receive, MAIT cells could have different roles during the development of neuroinflammation.

To translate our results into a clinical setting, we characterized MAIT cells in pwMS and HCs. In pwMS, we observed lower numbers of MAIT cells in both the peripheral blood and the CSF, together with a defective activation of MAIT cells after TCR stimulation with *E. coli*. Our observation of a decreased number if MAIT cells also in the CSF and not only in the peripheral blood of pwMS is against the hypothesis of their migration inside the CNS during neuroinflammation. This is in contrast with previous studies demonstrating the presence of MAIT cells in MS demyelinating lesions[100], albeit in low numbers. This inconsistency could have two possible explanations. First, a limitation of our study is the lack of histologic staining on demyelinating lesions samples obtained from pwMS and HCs. Thus, we cannot exclude that MAIT cells accumulate inside the CNS using an alternative route of entry. Secondly, Willing and collaborators used CD8 and CD161 as markers to identify MAIT cells in the brain parenchyma[100]. Tha family of CD161-expressing $CD8^{+}$ T cells includes not only MAIT cells, but also cytotoxic Tc17 cells and stem cell like T memory cells[135]. Contrary to other studies[104] we couldn't confirm an increased production of IL-17A by MAIT cells obtained from the peripheral blood of pwMS, albeit we did describe a trend in this sense. It's possible that a subset of MAIT cells, producing IL-17A is infiltrating the CNS system using alternative routes of entrance in pwMS, producing high amount of IL-17A directly at the site of lesions.

Furthermore, we observe a defective suppression of in vitro $CD4^+$ T cell proliferation by MAIT cells obtained from pwMS. The effect of T cell activation on $CD4^+$ and $CD8^+$ T cells has not been extensively studied. We described an inhibitory effect of MAIT cell activated through specific TCR stimulation by *E. coli*. *E. coli* stimulation should induce MAIT cells to produce mainly IFN-γ, but it's possible that a parallel production of granzyme is responsible for the suppression of the proliferation.

It's not known if also in humans MAIT cell phenotype is determined by the gut microbiome composition during the first years of life but given the high level of evolutionary conserved homology in MR1 sequences among mammalian species, it's prudent to suppose so. The alterations we have observed in MAIT cells in pwMS could be explained by an altered gut microbiome composition, determining either a preferential sequestration of MAIT cells in the intestine lamina propria and/or an exhaustion and defective activation of MAIT cells. We have previously described an overrepresentation of the fungus genera *Saccharomyces* and *Aspergillus* in the gut microbiome of pwMS compared to HCs[129]. Like the bacteria *E. coli*, the fungi *Saccharomyces* and *Aspergillus* are riboflavin producer and could activate MAIT cells both in vitro and in vivo[136]. In response to acute activation by the gut microbiome MAIT cells upregulated activation markers like PD1, homing molecules, like CCR6 and CCR9 and produce high amounts of pro-inflammatory cytokines, like IFN-γ[96]. Chronic activation can lead to exhaustion and defective cytokine production after TCR stimulation. Our observation of an upregulation of the riboflavin metabolic pathway in the serum of pwMS compared to HCs further support this hypothesis.

A lot of controversy about the number and the phenotype of MAIT cells in pwMS still exists. It has been demonstrated that MAIT cell numbers and phenotype are influenced by age, tobacco use and certain drugs[103]. All these factors could explain the variability observed in previous studies.

Obviously, our results warrant further investigation and confirmation but the possibility to manipulate MAIT cell function just by influencing the gut microbiome composition is extremely appealing from a therapeutic point of view.

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