

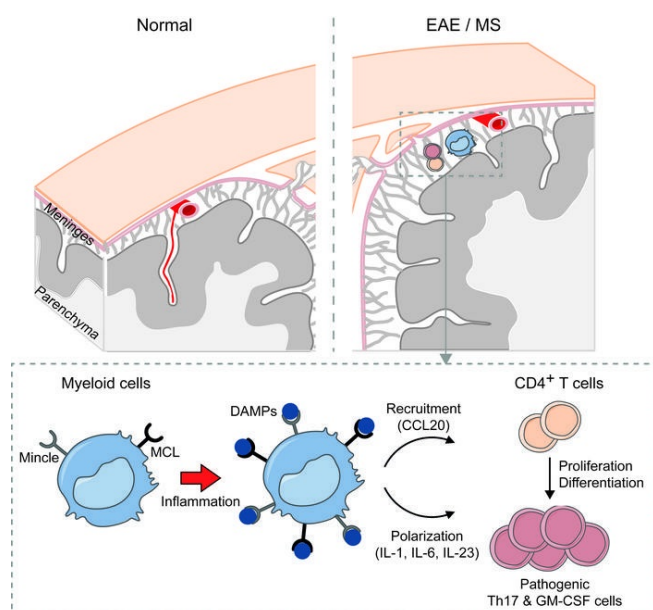
C-type lectin receptors Mcl and Mincle control development of multiple sclerosis-like neuroinflammation

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Title: C-type lectin receptors Mcl and Mincle control development of multiple sclerosis-like neuroinflammation

One Sentence Summary: The C-type lectin receptors Mcl and Mincle modulate neuroinflammation by altering myeloid cell activation and thereby T helper cell recruitment, reactivation and fate in the CNS.

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Conflict of interest:

The authors have declared that no conflict of interest exists.

Keywords

Multiple Sclerosis, EAE, autoimmunity, C-type lectin receptors, MCL, Mincle, SAP130

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Abstract:

Pattern recognition receptors (PRRs) are crucial for responses to infections and tissue damage, however, their role in autoimmunity is less clear. Herein we demonstrate that two C-type lectin receptors (CLRs), Mcl and Mincle, play an important role in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), an animal model of Multiple Sclerosis (MS). Congenic rats expressing lower levels of Mcl and Mincle on myeloid cells exhibited a drastic reduction in EAE incidence. In vivo silencing of *Mcl* and *Mincle* or blockade of their endogenous ligand SAP130 revealed that receptors expression in the central nervous system is crucial for the T cell recruitment and reactivation into a pathogenic Th17/GM-CSF phenotype. Consistent with this, we uncovered MCL/MINCLE-expressing cells in brain lesions of MS patients and we further found an upregulation of the MCL/MINCLE signaling pathway and an increased response following MCL/MINCLE stimulation in peripheral blood mononuclear cells from MS patients. Together these data support a role for CLRs in autoimmunity and implicate the MCL/MINCLE pathway as a potential therapeutic target in MS.

Main text:

Introduction

Multiple Sclerosis (MS), a leading cause of neurological disability in young adults, is a chronic inflammatory disease of the central nervous system (CNS) characterized by autoimmune destruction of myelin and subsequent neuro-axonal loss. Autoreactive CD4⁺ T cells infiltrating the CNS are considered to be important triggers of MS pathogenesis (1). Antigen presenting cells (APCs) play a crucial role in the reactivation of CD4⁺ T cells, which drive the subsequent pathogenic inflammatory responses (2-4). However, the mechanisms by which T cell fate is determined upon reactivation in the CNS remain poorly understood. A growing body of evidence suggests a role for pattern recognition receptors (PRRs) in autoimmune inflammatory responses (5). Previous studies have primarily focused on toll-like receptors (TLRs), showing their potential role in modulating pathogenic disease pathways (6-8), however, little is known about the role of C-type lectin receptors (CLRs) in autoimmunity and particularly in MS (9).

Myeloid CLRs are important sensors of infectious agents and tissue homeostasis through recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (10, 11). In addition to their well-known role in infections and tissue damage, recent animal studies have implicated several CLRs as risk genes for autoimmune diseases including arthritis and experimental autoimmune encephalomyelitis (EAE), an MS-like disease model (12, 13). Among the CLRs, CLEC4D (MCL) and CLEC4E (MINCLE), two members of the Dectin-2 family primarily co-expressed on myeloid cells (14), have recently gained attention in human inflammatory diseases. MCL and MINCLE signaling leads to the activation of NF- κ B via the BCL10-CARD9-MALT1 pathway (11, 15). Interestingly, several members of this pathway have been identified as candidate risk genes for multiple chronic inflammatory diseases such as Crohn's disease, ankylosing spondylitis, ulcerative colitis, inflammatory bowel disease and MS (16, 17).

In the present study, we investigated the role of Mcl and Mincle in EAE and MS. Myelin oligodendrocyte glycoprotein (MOG)-induced EAE in rats provides a robust model of chronic

relapsing-remitting disease, recapitulating several clinical and histopathological features of MS (18). We exploited the fact that the Dark Agouti (DA) strain is highly susceptible to EAE while the Piebald Virol Glaxo (PVG) strain is relatively resistant (18), and thus generated a congenic strain containing a PVG region encompassing CLR genes on the DA background. Herein we report that this new strain exhibits reduced expression of Mcl and Mincle on myeloid cells and is resistant to EAE induction. Mechanistically, we demonstrate that meningeal myeloid cells orchestrate the recruitment of T cells to the CNS and their reactivation into a pathogenic Th17/GM-CSF phenotype via the Mcl/Mincle pathway. This mechanism is likely triggered, at least in part, by the alarmin SAP130 during early disease development. Both MCL and MINCLE are highly expressed on infiltrating macrophages in active MS lesions. Notably, we show that the MCL/MINCLE pathway is affected in MS, with high expression of the receptors on immune cells associating with both increased MS disease activity and progression, and that MCL/MINCLE stimulation *ex vivo* results in higher cytokine production in peripheral blood mononuclear cells (PBMCs) of MS patients compared to healthy controls.

Results

The CLR gene expression in immune cells modulates EAE development

We have previously shown that a genetic region on rat chromosome 4 encompassing a cluster of CLRs confers resistance to EAE when transferred from the resistant PVG strain to the susceptible DA strain (12, 19). In order to identify the gene(s) responsible for this protective effect, we have further reduced the size of the transferred region in a congenic strain (hereafter referred to as CLRC) comprising five genes, four of which belong to the Dectin-2 family (Table S1). Upon MOG immunization, the homozygous CLRC rats displayed marked protection compared to DA homozygous littermate controls, while heterozygous rats (Het) exhibited an intermediate phenotype (Figure 1A). In accordance, weight loss in CLRC was significantly milder, recovery faster, disease incidence as well as average, cumulative and max scores lower compared to DA rats (Figure 1A). Histopathological investigation of the spinal cord at day 29 post-immunization (p.i.) revealed significant reduction in both extent of demyelination and inflammatory index in CLRC rats (Figure 1B and Figure S1A). Interestingly, only DA rats presented demyelination in the brain while this was not observed in either CLRC or Het rats (Figure 1B and Figure S1B). These results indicate that the PVG alleles encompassing the CLR genes confer resistance to EAE.

We next examined the contribution of CLRs in CNS- versus bone marrow (BM)-derived immune cells using bone marrow chimeric rats. Reconstitution of lethally irradiated DA or CLRC rats with BM isolated from DA-GFP rats led to the replacement of peripheral immune cells, as well as meningeal and perivascular macrophages with donor BM (Figure S1C-F). No significant differences in EAE development, weight change, incidence or average cumulative and max score were observed between the strains (Figure 1C). However, the transplantation of DA-GFP rats with DA or CLRC bone marrow recapitulated the phenotype observed in congenic animals, with CLRC->DA-GFP chimeric rats displaying significantly reduced disease incidence, weight and average, cumulative and max score compared to DA->DA-GFP chimeras (Figure 1D, Figure S1G and H).

These findings suggest that the protective effect of the CLRC congenic is mediated through the peripheral immune cell compartment and/or the meningeal/perivascular macrophages and not via the CNS-resident microglia.

The CLRC locus modulates activation of peripheral T cells recruited to the CNS

Myeloid antigen-presenting cells play a pivotal role in T cell activation and subsequent EAE progression (2-4). As C-type lectins are primarily expressed on myeloid cells (20) we first investigated whether the lower EAE incidence in CLRC rats resulted from an incomplete activation of T cells during the priming phase. Characterization of draining lymph nodes at day 7 p.i. revealed no differences in CD4⁺ T cell infiltration, activation, cytokine production or proliferation, implying that peripheral priming is unaffected in CLRC congenic rats (Figure 2A-C, Figure S2A and B). However, analysis of cellular infiltration in the spinal cord at disease onset (day 13 p.i.) revealed reduced numbers of CD4⁺ T cells, monocytes/macrophages and granulocytes in CLRC compared to DA rats (Figure 2D and Figure S2C). Further characterization of the infiltrating CD4⁺ T cells revealed a significant reduction of Ki67⁺ cells, a marker of proliferation (Figure 2E), along with reduced proportions of IL-17, IL-17/IFN γ double producers and GM-CSF-producing T cells (Figure 2E and F), regarded as the most inflammatory T cell phenotypes in the context of EAE (21-26). No significant differences were observed in IL-10 and IFN γ production (Figure 2E and F). Evaluation of meninges and the spinal cord prior to clinical signs of EAE revealed a 2- to 4-fold more extensive infiltration of macrophages, monocytes, granulocytes and CD4⁺ T cells in the meninges compared to the spinal cord (Figure S1I), regardless of the genotype. This finding strengthens the notion that the most likely site for reactivation of antigen-specific T cells is in the meninges, as previously suggested (3, 4).

Overall, these results suggest that CLRs modulate the reactivation of T cells within the CNS, most likely in the meninges, and participate in the differentiation or maintenance of IL-17- and GM-CSF-producing cells.

Reduced expression of *Mcl* and *Mincle* in monocytes/macrophages associates with protection from EAE

Using sequencing and single nucleotide polymorphism typing, we determined that the CLRC congenic region contains four genes of the CLR Dectin-2 family, i.e. *Dcar1*, *Mcl*, *Mincle* and the pseudogene *Dectin-2p*, as well as the vomeronasal gene *Vom2r48* (Table S1). We first examined the association of genes of the CLRC locus with different EAE disease parameters using gene expression data from splenocytes of (DAXPVG)xDA backcross rats (n=150) subjected to EAE (27). Among the five investigated genes, significant associations with disease phenotype were only observed for *Mcl* and *Mincle* (Table S2). The expression of both genes positively correlated with disease incidence (Figure 3A), maximal score, cumulative score, disease duration, terminal score and maximum weight loss (Table S2 and Figure S3). We next assessed the expression of the CLRC genes in splenocytes of naïve DA and CLRC rats by qPCR and determined a significantly reduced expression of *Mcl* (70%) and *Mincle* (85%) and increased expression of *Dcar1* (212%) in CLRC animals compared to DA rats (Figure 3B). Expression of *Vom2r48* was below the detection threshold and we failed to amplify *Dectin-2p* despite employing multiple strategies, which is not unusual for a pseudogene. Since the expression of *Dcar1* did not correlate with any phenotypic features of disease, the reduced expression of both *Mcl* and *Mincle* in immune cells is more likely to confer the protection to EAE observed in CLR congenic rats.

To further characterize the cellular compartment mediating the EAE phenotype, we examined expression of *Mcl* and *Mincle* on different immune cells in DA and CLRC rats. In the blood of naïve animals, significantly fewer circulating *Mcl*⁺ and *Mincle*⁺ monocytes were observed together with reduced surface receptor expression on both monocytes and granulocytes in CLRC compared to DA rats (Figure 3C and Figure S4). In the spinal cord, naïve CLRC rats displayed a reduction of both frequency of *Mcl*⁺ and *Mincle*⁺ cells and relative receptor expression levels on infiltrating monocytes/macrophages. Similar results were observed in both the blood and spinal cord of EAE affected animals, although only *Mincle* and not *Mcl* expression was affected in granulocytes during disease (Figure 3C and Figure S4). No difference in the microglial compartment was observed in EAE

animals, supporting the hypothesis that expression of the receptors on microglia is not likely to affect EAE disease outcome (Figure 3C and Figure S4).

To analyze the effect of CLRC on tissue-resident and inflammatory myeloid cells we generated bone marrow-derived macrophages (BMMa) and dendritic cells (MoDC) (28), and observed reduced expression of *Mcl* and *Mincle* in CLRC cells compared to cells derived from DA rats (Figure 3D). Furthermore, *Mcl* and *Mincle* expression in the meninges ex vivo was significantly lower in naïve and immunized CLRC rats (Figure 3D). This was reflected at the protein level, where 11 days p.i. receptor expression was only detected in DA rats but not in CLRC spinal cord meninges (Figure 3E), in concordance with *Mcl*⁺ and GFP⁺ cells only being detected in the meninges of DA-GFP → DA rats (Figure S1J).

Taken together, these data indicate that the EAE protection conferred by the CLRC locus is associated with reduced expression of *Mcl* and *Mincle*, particularly in myeloid cells residing in the meninges.

Impaired response to *Mcl*/*Mincle* ligands in CLRC myeloid cells affects CD4⁺ T helper cell recruitment and skewing

To further investigate the functional consequences of low *Mcl*/*Mincle* levels, we assessed qPCR expression of genes known to be induced by the *Mcl*/*Mincle* signaling pathway in BMMa, MoDC and FLT3-derived bone marrow dendritic cells (BMDC) stimulated with *Mcl*/*Mincle*-specific ligands, TDM and TDB. All CLRC myeloid cell types showed a reduced expression of *Mcl*/*Mincle* (Figure S5A-C) and an impaired response to *Mcl*/*Mincle* ligand activation (Figure 4A and Figure S5D-E). More specifically, CLRC BMMa expressed significantly less cytokines such as *Il1b*, *Il6*, *Il23*, *Il12p40*, *Tnf* as well as the chemokines *Ccl2*, *Ccl3*, *Ccl20*, *Cxcl1* and *Cxcl2* upon stimulation with TDM and/or TDB compared to DA cells. Similar results were obtained when stimulating MoDCs and BMDCs (Figure S5D and E). We also investigated the signals that regulated *Mcl*/*Mincle* expression and found TNF, and to a minor extent IL-1α and IL-1β, to upregulate both receptors (Figure S5G).

T helper cell differentiation requires TCR activation (signal 1), co-stimulation (signal 2) and a third signal mediated by cytokines. We therefore performed in vitro T cell differentiation assays using the supernatants from TDM- or LPS-stimulated MoDCs as a surrogate for the third signal of T cell activation. While we did not detect any differences in cytokine production or proliferation of CD4⁺ T cells exposed to DA or CLRC supernatants from LPS-stimulated cells, supernatants from TDM-stimulated CLRC cells led to a reduced induction of IFN γ and IL-17 together with a strong decrease in cell proliferation accompanied by increased IL-10 production (Figure S5F). This reduced inflammatory profile did not stem from either TGF β or IL-10 production by TDM-stimulated CLRC cells, since no difference in TGF β and only very low production of IL-10 could be detected in the MoDC supernatants (Figure S5H).

We thereafter explored whether the compromised response to Mcl/MinCLE stimulation in myeloid cells could affect T cell recruitment, activation and fate. We generated GFP⁺ MOG-specific resting effector T cells (GFP⁺ MOG-T_{eff}). GFP⁺ MOG-T_{eff} cells were reactivated in vitro with either DA or CLRC BMMA to mimic the reactivation of pathogenic T cells in the CNS (Figure 4B). Co-culture of GFP⁺ MOG-T_{eff} with CLRC BMMA led to a reduced proliferation of T cells as well as reduced number of IFN γ -, IL-17- and GM-CSF-producing T cells compared to cells cultured with DA BMMA. We then assessed if CLRC BMMA could promote differential recruitment of GFP⁺ MOG-T_{eff} across a CNS vascular endothelial cell layer, mimicking the meningeal blood-CSF barrier, using a transwell migration assay. We found higher retention of GFP⁺ MOG-T_{eff} in the upper chamber (higher ratio) when T cells were exposed to CLRC BMMA, whereas DA BMMA induced more extravasation through the endothelial layer (lower ratio) (Figure 4C). Finally, we transferred the GFP⁺ MOG-T_{eff} cells into immunized DA or CLRC rats day 6 p.i. and assessed T cell frequencies and activation at disease onset (day 13 p.i.). Similar to EAE induction in un-manipulated DA and CLRC rats (Figure 2), we observed less proliferation as well as less IL-17- and GM-CSF-producing GFP⁺ T cells in the spinal cord of CLRC rats compared to DA (Figure 4D). Interestingly, we also recovered less GFP⁺ MOG-T_{eff} in the CNS of CLRC rats and those cells proliferated less and also produced less IL-17 and GM-CSF (Figure 4D).

Altogether, our in vitro and in vivo findings indicate that reduced Mcl/Mincle expression results in impaired myeloid cell activation that leads to reduced T cells recruitment to the CNS and compromised T helper cell reactivation.

Downregulation of Mcl and Mincle, and their endogenous ligand SAP130, contributes to EAE pathogenesis

To confirm the impact of Mcl and Mincle expression on neuroinflammation in EAE, we performed in vivo CNS-specific knockdown of *Mcl* and *Mincle* expression. Infiltrating and resident cells in both spinal cord and the brain subarachnoid space were targeted via delivery of *Mcl/Mincle*-specific siRNA using intrathecal (i.t.) and intracisternal (i.c.) injections at days 7, 9 and 12 p.i. in disease-susceptible DA rats. The silencing efficiency of combined siRNA targeting of *Mcl* and *Mincle* was verified in vitro in bone marrow-derived macrophages (BMMa), with a 50-70% decrease of Mcl and Mincle surface expression compared to control siRNA (Figure S6). Interestingly, rats treated with *Mcl/Mincle*-siRNA had a reduced EAE disease incidence resulting in lower cumulative and average scores as well as weight decrease compared to animals treated with control siRNA (Figure 5A). This was accompanied by a trend of decreased infiltration of granulocytes and reduced frequency of IL-17- and GM-CSF-producing CD4⁺ T cells in the CNS prior to disease onset (Figure 5B and C). In rats treated with either *Mcl*- or *Mincle*-specific siRNA separately, we also observed a protection that was less robust compared to the effect of combined targeting of *Mcl* and *Mincle* (Figure 5A). Altogether, the results validate that altered expression of Mcl and Mincle modulates EAE susceptibility by affecting T cell reactivation in the CNS.

Mcl and Mincle can recognize both PAMPs and DAMPs. The danger molecule SAP130 was the first established endogenous ligand demonstrated to bind to Mincle (15) and elevated SAP130 levels have been implicated in several inflammatory experimental models (29, 30). We addressed the possible involvement of SAP130 in disease and hypothesized that blocking the protein in susceptible rats would affect EAE pathogenesis. Immunized DA rats were treated i.t. and i.c. with either anti-SAP130 or isotype control antibodies at day 2 and 7 p.i. Remarkably, blocking SAP130 in the target organ in

vivo led to a reduction in EAE score as well as weight loss (Figure 5D). Additionally, a tendency for reduced incidence, average and cumulative score was observed.

Taken together, these results suggest that Mcl, Mincle and their putative ligand SAP130 promote EAE pathogenesis.

Increased activity of the MCL and MINCLE signaling pathway in MS patients

Our findings implicate a role for the C-type lectins Mcl and Mincle in the pathogenesis of EAE. To explore their potential relevance in human disease we first examined receptor expression in MS brain tissue both in active and chronic active lesions (Table S3). Interestingly, both MCL and MINCLE expression was detected in active lesions and in the rim of chronic active lesions. Immunoreactivity of MCL and MINCLE localized on HLA-DR immunopositive cells, indicating expression on antigen-presenting cells, primarily myeloid cells (Figure 6A and Figure S7A). We thereafter determined the expression of genes involved in the MCL/MINCLE signaling pathway in circulating blood cells by analyzing the transcriptome of PBMCs in a large cohort of MS patients, patients with clinically isolated syndrome (CIS) and non-inflammatory neurological disease controls (NINDCs) (Table S4). In accordance with previous studies reporting co-regulation of *MCL* and *MINCLE*, we could detect a strong correlation between *MCL* and *MINCLE* expression in PBMCs from MS patients (Figure S7B). Gene expression analysis revealed a significant increase in *MINCLE* and one of the downstream mediators, *CARD9*, in CIS patients and RRMS patients during relapse compared to NINDCs (Figure 6B, Figure S7C).

We further investigated a putative association between the MCL/MINCLE pathway and MS disease severity by stratifying MS patients according to level of accumulated disabilities (Expanded Disability Status Scale, EDSS). We observed higher expression of *MINCLE*, *CARD9* and *IL8* in patients that have higher disabilities (EDSS>4.5) compared to less affected patients (Figure 6C). Together, these results indicated a link between increased peripheral inflammatory activity and disease severity with the MCL/MINCLE signaling pathway in MS patients and prompted us to explore the impact of

MCL/MINCLE activation in immune cells. To this end, we stimulated PBMC isolated from RRMS patients during relapse and from healthy controls (Table S5) with either TDB or LPS and examined the modulation of *MCL/MINCLE* expression as well as the downstream mediators, *SYK*, *MALT1* and *CARD9*, and effector cytokines IL-8, IL-6 and TNF (Figure 6D and E, Figure S7D-G) (31). Interestingly, TDB stimulation resulted in a significant increase of IL-8 production both at mRNA and protein levels, but not IL-6 or TNF, in monocytes from MS patients compared to healthy controls. Of note, there was no difference in *MINCLE* basal expression prior to stimulation between MS and HC monocytes, but there was an increased expression of *MCL*, *SYK* and *MALT1* in MS patients (Figure S7D-E). Upon TDB stimulation, *MCL*, *MINCLE* and *MALT1* were significantly upregulated in monocytes from MS patients (Figure S7D-E), with a similar trend observed for *SYK* and *CARD9*.

Altogether, our data strongly suggest an increased activity of the MCL/MINCLE signaling pathway in MS patients, which could play a role in the inflammatory response and disease progression.

Discussion

Using a congenic rat strain with natural variation in expression of C-type lectin receptors, we investigated the role of PRRs Mcl and Mincle in EAE pathogenesis, and further examined the findings in human disease. Our data demonstrate that lower expression of Mcl and Mincle in the congenic strain protects them from development of MOG-EAE. Reduced receptor expression drastically affects the response of antigen-presenting cells to specific ligands and mitigates their ability to recruit and support pathogenic Th17/GM-CSF phenotype. Moreover, we determined that MCL and MINCLE are expressed in MS lesions, that the signaling pathway is active in PBMCs of CIS and MS patients during relapse compared to controls, and that monocytes from MS patients responded more vigorously to specific receptor stimulation. Furthermore, expression of genes in the MCL/MINCLE pathway was increased in patients with higher disabilities, suggesting a role for this pathway in disease progression.

While PRRs, especially *MINCLE*, have been implicated in peripheral inflammatory disorders such as rheumatoid arthritis (32, 33), their role in CNS-related immune diseases such as MS remains elusive. Peripheral inflammation induces substantial changes in the CNS, particularly local neuroinflammation mediated by glial activation and subsequent production of pro-inflammatory factors such as TNF, IL-1 β and IL-6 cytokines (34, 35). Accordingly, studies have shown that following peripheral inflammation, recruitment of T cells and monocytes into the CNS and the resulting tissue damage is strongly influenced by local production of IL-6 and its effect on blood brain barrier permeability (36, 37). Our data demonstrate that regulation of Mcl and Mincle in meningeal myeloid cells modulates T cell recruitment and reactivation in the CNS by a number of mechanisms that might include both pro-inflammatory cytokine and chemokine production, suggesting that these receptors would play a role in initiating or amplifying immune responses in the target organ. This is further supported by the increased expression of *MCL*, *MINCLE* and down-stream mediator *CARD9* observed in PBMCs of MS patients during active disease. Furthermore, we found that the MCL/MINCLE ligand TDB induces higher production of IL-8 by PBMCs from MS patients compared to controls, which is in accordance with previous data implicating the downstream mediators, *CARD9* and *SYK*, in IL-8 production by human myeloid cells following TDB stimulation (31). Interestingly, animals with

MOG₃₅₋₅₅-specific CD4⁺ T cells (2D2 mice) display a higher incidence of spontaneous EAE disease following influenza respiratory infection when compared to non-infected mice, suggesting that the peripheral infection can trigger CNS immunosurveillance and disease (38). Consistent with this, epidemiological data have suggested that systemic infection either precedes or concurs with disease exacerbation in MS patients (39) and that stress may also be a contributing factor (40). Hence, stress or infections could induce expression of PRRs in CNS innate cells of MS patients, promoting peripheral immune cell CNS patrolling, which would result in disease relapse. The protective effect of i.t and i.c. siRNA-mediated knockdown of *Mcl* and *Mincle* strongly supports a role for the receptors in the regions directly in contact with the CSF, such as the leptomeninges. Our findings demonstrating the expression of *Mcl* and *Mincle* in meningeal macrophages of EAE animals and receptors expression in MHC II⁺ cells in brain lesions of MS patients argues in favor of this hypothesis. Taken together, these data point to a possible role of CNS-intrinsic processes involving MCL, MINCLE, and their signaling pathways during the inflammatory phase of MS.

Our data provide in vitro and ex vivo evidence that *Mcl* and *Mincle*, expressed by monocyte-derived cells, macrophages and/or dendritic cells, shape the activation towards a Th17 and GM-CSF-producing T cell phenotype in EAE. While we also see an increased influx of granulocytes upon disease onset, the effect of these cells in further shaping the inflammatory response in the CNS is most likely secondary to T cell recruitment and activation, as it has been shown that neutrophils only acquire significant antigen-presenting capacity upon exposure to T cell-derived cytokines (41). Our findings concord with previous studies reporting an effect of *Mincle* on T helper cell differentiation, notably of Th17 cells (42), and a *Mincle*-associated Th17 response in vivo in the context of autoimmunity and sterile inflammation (43, 44). Notably, the mechanism of action for *Mcl* and *Mincle* appears highly important in light of the crucial role of the Th17 and the cytokine GM-CSF in MS pathogenesis (21-26). Indeed, previous studies have determined an increased frequency of Th17 and GM-CSF-producing Th cells in the blood and CSF of MS and CIS patients during active disease (45, 46), and have further associated the frequency of effector memory Th17 cells as well as IL-17a levels

with disease severity (EDSS) (45, 47). Similarly, we found that expression of genes from the MCL/MINCLE signaling pathway correlates with disease activity and severity.

The danger theory, proposed by Matzinger and colleagues (48), suggests that the immune system responds to intrinsic and extrinsic danger signals (PAMPs and DAMPs) by recognition through PRRs. DAMPs, sometimes referred to as alarmins, are endogenous molecules released upon cellular activation, stress or damage and can induce activation of inflammatory pathways through PRRs in the absence of infection. Accordingly, several DAMPs have been implicated in the pathogenesis of autoimmune diseases such as RA, SLE or systemic sclerosis (49, 50). Among the established endogenous ligands of Mincle, SAP130 has been reported to contribute to neuroinflammation in models of cerebral hemorrhage and traumatic brain injury (30, 51). Herein we showed that blocking the SAP130 in the CNS ameliorated EAE, suggesting that SAP130 can act as an alarmin in neuroinflammation. However, whether SAP130 or other endogenous ligands play a role in MS pathogenesis remains to be explored.

In conclusion, our study reveals an important role of the myeloid CLRs-mediated pathway in the amplification of autoimmune neuroinflammation upon initial CNS stress. In the future it will be crucial to define cellular and molecular drivers of the CLRC-mediated response using transgenic models as well as clinical samples in the context of genetic variants impacting the pathway. Of interest are also the mechanisms underlying CLRs upregulation in MS patients and the characterization of the alarmins or other metabolic mediators that may impact the sterile inflammatory response. This is of particular importance as most of the successful therapies for MS are able to prevent immune infiltration into the CNS but are limited in affecting subsequent steps of autoimmune attack in the target tissue.

Methods

Experimental design

The aim of this study was to investigate the potential role of pattern recognition receptors in autoimmune neuroinflammation, more specifically the role of the C-type lectin receptors Mcl and Mincle in multiple sclerosis, using both congenic rats expressing reduced levels of the receptors for in vivo and in vitro modelling as well as human samples from multiple sclerosis patients. For studies involving rats, littermate controls were used and in the event of treatment intervention, animals were randomly assigned into groups. In vivo and in vitro experiments were performed twice unless otherwise stated. For experiments involving human specimens, the number of samples included in each experiment was based largely on availability. Once experimental conditions were optimized, all data were included in the analysis. Data was thereafter only excluded upon technical experimental error. Animal experiments were performed blinded, as well as the initial analysis of ex vivo and in vitro experiments.

Animals

Inbred DA and PVG.1AV1 rats were originally obtained from the Zentralinstitut für Versuchstierzucht (Hannover, Germany) and Harlan UK Ltd (Oxon, UK) respectively. Separate lines have been established at Karolinska Institutet (DA/Kini and PVG.1AV1/Kini) (52). The CLRC congenic strain stems from a natural recombination event between the DA strain and the APLEC strain, encompassing a larger congenic fragment from the PVG background (19, 53). Transgenic eGFP DA rats have been described previously (54, 55). Experiments were performed with both male and female rats ranging between 8 and 16 weeks of age, and experiments were always carried out with sex- and age-matched animals. All animals were bred and kept in 12 h light/dark- and temperature-regulated rooms. Rats were housed in polystyrene cages containing aspen wood shavings and had access to standard rodent chow and water *ad libitum*.

Participants

Three cohorts of human samples were used in the study. Post-mortem brain samples of both active lesions and normal-looking white matter were collected from MS patients in the Netherlands brain bank. PBMCs from clinically-isolated syndrome (CIS) patients, untreated MS patients; relapsing-remitting in either relapse or remission, secondary progressive, primary progressive, as well as non-inflammatory neurological disease controls (NINDC) were isolated for whole genome RNA-sequencing. For the human in vitro experiments, natalizumab treated relapsing-remitting MS patients in remission and healthy controls were included.

Genotyping

Genomic DNA was extracted using alkaline lysis of ear or tail biopsies. TaqMan single nucleotide polymorphism (SNP) assay was used to determine the genotype according to the manufacturer's protocol (ThermoFisher).

EAE induction and experimental design

EAE induction. EAE was induced as previously described (18). Rats were anesthetized with isoflurane (Forene) and injected subcutaneously in the dorsal tail base with 200 μ l inoculum containing MOG (females 4-6 μ g and males 10-15 μ g) in PBS (Sigma-Aldrich) emulsified 1:1 with incomplete Freund's adjuvant (Sigma-Aldrich). Rats were weighed and monitored for clinical signs of EAE daily from day 7 until termination of the experiment. Clinical scoring was performed according to a standard scale for EAE: 0, healthy; 1, tail weakness or tail paralysis; 2, hind leg paresis or hemiparesis; 3, hind leg paralysis or hemiparalysis; 4, tetraplegy and 5, death. The following clinical parameters were assessed: incidence of EAE (clinical signs of EAE for at least two consecutive days), onset of EAE (the first day of clinical signs), maximum EAE score (the highest clinical score),

cumulative EAE score (the sum of daily clinical scores), duration of EAE (number of days with EAE), weight change (calculated by subtracting the daily weight from the weight at day 7 and expressing the difference as a ratio of the weight at day 7) and maximum weight loss (calculated by subtracting the lowest weight during the experiment from the weight at day 7 and expressing the difference as a ratio of the weight at day 7).

BM chimeras. Eight-week-old recipient animals (n=7 or 8 per group) were lethally irradiated (2x550Gy) and injected i.v. with freshly isolated bone marrow (BM) cells (50×10^6 /300ul) from donor animals (n=3 per group). Rats were left to reconstitute for 8 weeks. Reconstitution efficiency was determined in blood by flow cytometry and animals were thereafter subjected to EAE.

Drug administration. Immunized rats were randomly divided into two groups (n=7 or 8 per group) and were treated with siRNAs, anti-SAP130 or respective controls by intrathecal (i.t.) and intracerebroventricular (i.c.) (via cisterna magna) injections with 10 µl per injection. Rats were injected under 3.5%-isoflurane anesthesia, as previously described (56). The injections were performed with a 50 µl Hamilton syringe (Sigma-Aldrich). Animals were monitored daily and clinical signs of EAE were recorded as described above. For the siRNA treatment, groups received i.t. and i.c. injections of siRNAs against *Mcl*, *Mincle*, both *Mcl* and *Mincle* or scramble siRNA, 5ug/10ul/injection in Accell siRNA delivery media (media and siRNA from Dharmacon) at day 7, 9 and 12 post immunization. For the anti-SAP130 treatment, both groups received i.t. and i.c. injections of either anti-SAP130 or rabbit-IgG (both from Abcam), 5ug/10ul/injection in PBS at day 2 and 7 post-immunization.

Tissue collection

Lymph nodes and bones for bone marrow extraction. Rats were sacrificed with CO₂ prior to dissection of inguinal lymph nodes and femoral bones and tissues were kept in PBS on ice until cell preparation.

CNS tissue. Rats were anesthetized with isoflurane and perfused with PBS containing Heparin at 2500 IU/I (Sigma-Aldrich) via the left ventricle. For flow cytometry, brains and spinal cords were excised

and placed separately in 37% Percoll solution (Sigma-Aldrich) containing 50 U/ml DNase I (Roche Applied Science) on ice until further processing. Meninges from the spinal cord were separated and collected in cold PBS containing 2mM EDTA (Sigma-Aldrich). For gene expression analysis, spinal cord or meninges were collected in RLT buffer containing dithiothreitol (DTT, Sigma-Aldrich), dissociated using a tissue lyser (Qiagen) and supernatants were collected and frozen at -70°C until RNA preparation. For immunohistochemistry, rats were further perfused with 4% paraformaldehyde (Histolab) and skulls and spines were dissected and kept in 4% PFA for 24hrs.

Single cell preparation and *ex-vivo* culture

Lymph nodes. Lymph nodes were mechanically dissociated and cells were spun and re-suspended in complete media, RPMI complemented with 5% FCS, 1% L-glutamine, 1% penicillin-streptomycin and 1% pyruvic acid (all from Sigma-Aldrich) and 50 mM 2-mercaptoethanol (Gibco-BRL). Subsequently, cells were plated either in 96-well V-bottom plates for further flow cytometry analysis or in 96-well U-bottom plates for recall stimulation (72 hours with 20µg/ml MOG) and thereafter flow cytometry analysis.

For in vitro T cell differentiation assay, LN single cell suspension were depleted of CD25⁺ cells (which include both recently activated T cells as well as Tregs) with anti-CD25-PE antibody followed by anti-PE beads and subsequently CD4⁺ cells were positively sorted using anti-CD4 beads (Miltenyi). Resting CD4⁺ T cells were added to 96-well flat plates pre-coated with anti-CD3 (1.25mg/ml, BD Pharmingen), and cultured with supernatant from stimulated MoDCs and soluble CD28 (1mg/ml, BD Pharmingen) for 4 days. Intracellular cytokine production was assessed by flow cytometry.

CNS cells. Brain and spinal cord were dissociated with a glass homogenizer in 37% Percoll solution and underlaid with 70% Percoll solution. After centrifugation at 1000 g for 30 min, the intermediate layer was carefully collected and further diluted in HBSS (Sigma-Aldrich) prior to a 15 min-centrifugation at 600 g. Cell pellets were re-suspended in PBS (Life Technologies) and further used for FACS analysis. Meninges were incubated at 37C for 20 min in PBS/EDTA and then mechanically

dissociated through a 40 μ m cell strainer. Cells were spun and resuspended in PBS until staining for flow cytometry.

BM-derived cells. Bone marrow was flushed out of femurs with PBS. BM single cell suspension was used for injection in BM chimera or plated to prepare BM-APCs. BM-APCs were prepared as described previously with modifications (28, 57). Briefly, BM cells were cultured in complete DMEM (Sigma-Aldrich) complemented with 5% FCS, 1% L-glutamine, 1% penicillin-streptomycin and 1% pyruvic acid (all from Sigma-Aldrich) and 50 mM 2-mercaptoethanol (Gibco-BRL), supplemented with either a combination of 20 ng/ml rat rGM-CSF and 5 ng/ml rat rIL-4 (to obtain MoDCs), with 200ng/ml rhFLt3L (to obtain BMDC) or with 20 ng/ml rat rM-CSF (to obtain BMMa) for 9 or 7 days respectively (all cytokines were purchased from PeproTech). BMMa and MoDCs (10^5 cells/well) were stimulated in a 48 well plate with either complete media (control well), 10 μ g TDM (Innaxon), 10 μ g TDB (InvivoGen), with 100ng/ml LPS or with various concentrations of cytokines (TNF, IL-1 β , IL-6, all from PeproTech). TDM or TDB were resuspended in isopropanol at 0.1 mg/ml and each well was coated with 100 μ l, and left to evaporate in the laminar-flow cabinet. Isopropanol was added to the control wells. Cell supernatants were used for T cell differentiation assay. Adherent cells were lysed in RLT containing DTT for gene expression analysis. For Immunofluorescence (IF), DA-GFP BMMa were plated in culture slides and stimulated with cytokines for different time points. Cell were immediately fixed with 4% PFA and stained for IF.

In vitro siRNA-mediated MCL/Mincle silencing. *Mcl* and *Mincle* expression were reduced using siRNA in vitro. *Mcl* and *Mincle*-specific siRNAs or control scramble siRNA (Dharmacon) were prepared immediately prior to administration in Accell siRNA delivery media (Dharmacon), following the manufacturer's instructions. BMMa cells (10^5 cells/well in a 48-well plate) were treated with 6 μ g of scrambled siRNA, pooled *Mcl*- and *Mincle*-specific siRNAs (3 μ g each) or 6 μ g scramble-GFP siRNA and harvested 72 hours later in PBS/EDTA prior to flow cytometry.

Generation of the GFP⁺ MOG₉₁₋₁₀₈-specific T cells. DA-GFP rats were immunized with 100 µg MOG₉₁₋₁₀₈ in 200 µg CFA. After 7 days a single cell suspension from draining LNs was restimulated with 20 µg/ml MOG₉₁₋₁₀₈ at a concentration of 1×10^7 T cells/ml in RPMI complemented with 5% FCS, 1% L-glutamine, 1% penicillin-streptomycin and 1% pyruvic acid (all from Sigma-Aldrich), 50 mM 2-mercaptoethanol (Gibco-BRL) for 4 days at 37°C and 5% CO₂. Dead cells were removed with Ficoll (Ficoll Paque Plus GE healthcare) and live cells cultured for 5 days at a concentration of 2×10^5 cells/ml in cRPMI with IL-2 (20% filtered MLA culture supernatant).

GFP⁺ MOG₉₁₋₁₀₈-specific T cells and BMMA co-culture. BMMA cells were plated at 6.25×10^3 cells/well in 96-well U-bottomed plates with 20 µg/ml MOG₉₁₋₁₀₈ and left to rest for 24 hours after which 2.5×10^5 MOG₉₁₋₁₀₈-specific T cells were added to each well and incubated at 37°C and 5% CO₂ for 4 days. All conditions were made in duplicates.

Transwell experiments

Endothelial cell culture. Endothelial cell culture followed a previously described protocol (58) with adaptations for rat brain endothelia. DA rat brains were extracted and kept in cold dissection media (HBSS supplemented with 7.5% BSA and 1% penicillin-streptomycin). Brains were processed to remove the cerebellum and optic nerves, bisected, forebrains were separated, forebrain meninges were then removed under a stereomicroscope and the myelin duvet removed from the forebrain interior to obtain the cortex. The cortex was transferred to L15 media containing Papain (Sigma-Aldrich) and DNase1 (Calbiochem), mechanically dissociated by trituration and left to digest for 1 hour at 37°C. The enzymatic reaction was stopped with HBSS containing 10% FCS. The pellet obtained was re-suspended in 1xHBSS containing 22% BSA and separated using density dependent centrifugation at 800g for 15 minutes. The resulting pellet containing the brain micro-vessels was washed and added to complete rat brain endothelial growth culture media (cRBEGM – Sigma Aldrich) containing 10% FCS, 1% penicillin-streptomycin, hFGF 5 ng/ml, hVEGF 20 ng/ml and rEGF 5 ng/ml and plated in a T75 flask pre-coated in type IV collagen 20 µg/ml and fibronectin 20 µg/ml (all from R&D) and left to

incubate at 37°C and 5% CO₂. After 12h and again after 8h the medium was refreshed with cRBEGM, the second time containing 4 µg/ml puromycin and left for 5 days at 37°C and 5% CO₂. The media was refreshed with cRBEGM containing 0.5 µg puromycin for another 7 days after which it was replaced with complete cRBEGM with no puromycin. Growth was monitored to check for endothelial cell confluence.

Transwell migration assay. Endothelial cells were detached and harvested using accutase 1x solution in PBS at a ratio of 1:1. Pelleted cells were resuspended in cRBEGM at a concentration of 3x10⁵ cells/ml. Transwell plate bottoms were seeded with 3x10⁴ BMMA cells from either DA or CLRC resuspended in complete DMEM and allowed to settle for 3 hours at 37°C and 5% CO₂. The transwell inserts were prepared by coating with 100 µl of fibronectin at 20 µg/ml for 3 hours at 37°C and 5% CO₂ and then seeded with 100 µl of 3x10⁵ cells/ml rat brain endothelial cells, mounted on the wells containing the macrophages and allowed to rest for 24 hours at 37°C and 5% CO₂ to achieve confluence. After incubation, 3x10⁶ MOG₉₁₋₁₀₈-specific T cells were added in complete RPMI to each insert and the wells were allowed to incubate for 16 hours at 37°C and 5% CO₂ after which media from the upper well and the lower well were taken and cells stained and run for flow cytometry.

Flow cytometry

The staining procedures were performed by following the manufacturers' protocols. Briefly, single cell suspensions were incubated in 2% mouse serum (to block Fc receptors), then washed and incubated with antibodies for cell surface expression. Mcl-A488 and Mincle-A647 antibodies were kindly provided by Michael Rory Daws' laboratory (University of Oslo, Oslo, Norway) and have been validated previously (20). All other antibodies were obtained from BD bioscience, eBioscience or ABDserotec: CD25 (clone OX-39), CD134 (clone OX-40), CD44 (clone OX-49), MHC II (RT1B clone OX-6), CD45 (clone OX-1, # 561588, BD), CD11b (clone WT.5, #562108 BD), CD4 (clone OX-35, # 565432), Granulocyte marker (clone RP-1, #550002 BD). Dead cells were excluded using yellow fluorescence Live/ death marker (Molecular Probes). All stainings, washing steps and

acquisition were performed in cell staining buffer (BioLegend). For intracellular staining, cells were stimulated for 5h with Phorbol 12-myristate 13-acetate (PMA) (50 ng/mL, Sigma-Aldrich), Ionomycin (1 ug/mL, Sigma-Aldrich) and Golgi Plug (1 ul/mL, BD Bioscience). Cells were blocked with 2% mouse serum and stained for cell surface markers with yellow fluorescence Live/ death marker (Molecular Probes) prior to washing, fixation and permeabilization. Cells were then stained for intracellular proteins with antibodies specific to GM-CSF (MP1-22E9, eBioscience #12-7331-82), IL-17a (eBio17B7, eBioscience #45-7177-82), IL-10 (A5-4 BD bioscience #562156), IFN γ (DB-1, BD bioscience # 562213) and Ki67 (B56, BD bioscience #561281). Acquisition was performed using a Gallios flow cytometer and results were analyzed using Kaluza flow analysis software (both from Beckman Coulter).

Quantitative real-time PCR and Microarray

qPCR. Total RNA was extracted from cell lysate in RLT containing DTT using an RNeasy Mini kit (Qiagen) with the QiaCube according to the manufacturer instructions. cDNA was subsequently synthesized using iScript kit (Bio-Rad Laboratories). Real-time quantitative PCR was performed on a BioRad CFX384/C1000 Real-Time Detection System with a three-step PCR protocol using SYBR green fluorophore. Relative quantification of gene expression was calculated using the standard curve method with fold expression normalized in relation to the endogenous control gene β -Actin or *HPRT*.

Microarray. Expression of *Mcl* and *Mincle* assessed by microarray in spleens of (DAxPVG)xDA F1 rats subjected to EAE, as previously described (27). The following settings for analysis were used: Summarization: probe logarithmic intensity error (PLIER) described in the Guide to probe logarithmic intensity error (PLIER) estimation (<http://www.affymetrix.com>); Background correction: PM-GCBG and Normalization: Global Median. The microarray data are available in MIAME-compliant (minimal information about a microarray experiments) format at the ArrayExpress Database (<http://www.ebi.ac.uk/arrayexpress>) under accession code E-MTAB-784.

Histopathological analyses and immunohistochemistry

Histopathological analysis was carried out using Luxol fast blue and hematoxylin-eosin staining, paraformaldehyde-fixed 3 to 5 mm thick paraffin embedded sections of the brain and spinal cord were dewaxed in xylol, rehydrated and stained with hematoxylin and eosin and Luxol fast blue to assess inflammation and demyelination, respectively. The inflammatory index (I.I.) and demyelination score (DM) were determined by the number and size of demyelinated lesions of each rat on an average of 10 complete brain and spinal cord cross-sections as previously described (59).

For immunohistochemical analyses of rat samples, whole spine from PFA perfused animals were collected and kept in PFA overnight. After washing with PBS, samples were placed in 20N EDTA to decalcify the bones for 2 weeks and then placed in 30% sucrose solution for 1 week prior to freezing and sectioning onto superfrost glass slides. After rehydration in PBS, sections were stained with fluoromyelin (Invitrogen) according to the manufacturer's protocol. They were then stained for microglia (anti-Iba-1, Wako chemical # 019-19741), astrocytes (anti-GFAP, Dako, #M0761) or T cells (anti-CD4, Biorad Serotec #MCA55A647) followed by secondary antibody staining. For Mcl (anti-clec4d, Abcam #ab175021) and Mincle (16E3, Novus Biological #NBP1-49311AF488) staining, sections were subjected to antigen retrieval in target retrieval solution low pH (Dako) prior to staining. All washing steps were performed in TNT buffer (0.1 m Tris-HCl, pH 7.5. 0.15 m NaCl. 0.05% (v/v) Tween 20).

Human studies

Immunohistochemical analysis. Brain samples (n=5) containing active and chronic active lesions were obtained from 3 MS patients in collaboration with the VUmc MS Centrum Amsterdam and the Netherlands Brain Bank. Active lesions were characterized by abundant infiltrated macrophages and microglial cell activation throughout the lesion and overt demyelination. Chronic active lesions were fully demyelinated and contain a rim of macrophages and activated microglia. Detailed clinical data are summarized in Table S3. Formalin-fixed paraffin-embedded tissue was stained as described

previously (60). In short, deparaffinization and antigen retrieval, sections were incubated overnight with anti-MINCLE or anti-MCL (1:100) and anti-HLA-DR (anti-LN3, 1:100) followed by incubation with Alexa-488/594-labeled secondary antibodies (1:200; Molecular Probes) and analyzed by confocal microscopy (Leica DMI 6000 SP8, Leica).

Expression analysis. PBMCs were collected from 102 MS patients, 28 clinically isolated syndrome (CIS) patients and 36 non-inflammatory neurological disease controls (NINDC), diagnosed with e.g. neuralgia, paresthesia, sensory symptoms, vertigo and tension headache, between 2001 and 2010 at the Neurology Clinic of the Karolinska University Hospital, Solna, Sweden (61). At the time of sample collection 14 relapsing-remitting MS (RRMS) patients were in relapse and 73 were in remission, and the remaining patients were diagnosed with SPMS (n=8) or PPMS (n=7). mRNA was extracted from samples, cDNA libraries were prepared using Illumina TruSeq kit (Illumina, San Diego, USA) and sequenced on an Illumina HiSeq 2000 machine, as previously described (61).

For the RNA sequencing, we obtained paired-end reads with a length of 100 bp with an average sequence depth of 36 million reads per samples. The reads were mapped to the H. Sapiens reference genome (NCBI v37, hg19) using STAR aligner and HTSeq tool was used to quantify counts per gene, applying the default parameters in each case. The Conditional Quantile Normalization (CQN) method was used to normalize the count datasets and to account for the GC content bias. Implementing a component-based analysis, we regressed out the effects of batch of RNA-Seq library preparation from the normalized (CQN) count data. Residual values obtained after batch-effect correction was used for differential expression. The RNA-seq data will be made available from the corresponding author upon request and signature of data transfer agreement.

Ex vivo PBMCs culture. RRMS patients treated with Natalizumab (n=9) and healthy donors (n=11) were recruited and PBMC were isolated by density gradient centrifugation using Ficoll-Paque plus (GE Healthcare, Fairfield, CT) and stored in liquid nitrogen until use. Upon thawing, cells were

washed, counted and resuspended in RPMI medium supplemented with 20% FCS, L-glutamine and Penicillin-Streptomycin. Cells were plated at 2×10^6 cells/ml and stimulated with 40 μ g plate-bound TDB, 50 ng soluble LPS or wells were treated with isopropanol prior to airdrying as negative control. Cells were cultured for 48 h, and treated with GolgiPlug (BD Biosciences) 5 h prior to termination of the experiment. Cells were stained for MCL (clone 9B9 #360204) and isotype (clone MPC-11, #400314) expression prior to in vitro stimulations (BioLegend). Upon stimulation, cells were stained with LIVE/DEAD stain (ThermoFisher Scientific, #L34976), CD14 (clone HCD14 #325608), IL-8 (clone E8N1 #511406), IL-6 (clone MQ2-13A5, #501114), TNF (clone Mab11, #502926) or isotype for cytokines (clone MOPC-21, # 400108) (BioLegend).

Statistics

The 1-way ANOVA with Dunnett's Multiple Comparison Test was used to compare area under curve (AUC) of clinical EAE course and weight change as well as RNA-seq data, when more than two groups were compared. The Kruskal-Wallis test with Dunn's Multiple Comparison Test was used to compare all EAE score-based variables (average, cumulative and max EAE score) and histopathology variables, when more than two groups were compared. The Unpaired 2-tailed t-test was used to compare AUC of clinical EAE course and weight change when two groups were compared. The Mann-Whitney U test was used to compare all EAE score-based variables (average, cumulative and max EAE score) as well as microarray, FACS, qPCR and ELISA data, when two groups were compared. The Chi-square test was used when analyzing the differences in EAE incidence. Values are expressed as mean $\pm$ SEM or box-plots with whiskers representing 5-95 percentile. The GraphPad Prism $\geq$ 5.0 software (GraphPad) was used for statistical analyses and p-values <0.05 were considered significant.

Study approval

All animal experiments in this study were performed in accordance with the guidelines from the Swedish National Board for Laboratory Animals and European Community Council Directive (86/609/EEC), and approved by the North Stockholm Animal Ethics Committee (Stockholms norra djurförsöksetiska nämnd, Stockholm).

The human studies were approved by the Regional Ethics Committee (Stockholm, Sweden). All subjects in the RNAseq and in vitro monocyte stimulations provided written informed consent. For the brain cohort, all donors, or their next of kin, had given informed consent for brain autopsy and use of their brain material and clinical information for research purposes.

Author contribution:

Designing research studies: Studies were designed by MN with contributions from SB, AOGC and MJ.

Conducting experiments and acquiring data: MN, SB, SF, LK, AW, MZA, EP, JMH, WE, FM, CHY, VM, JVH and AOGC participated in the different experiments performed in this study.

Analyzing data: MN, SB, MZA, TJ, ML, JVH, MJ and IK contributed to the analysis of the data.

Providing reagents: HMR, MRD, JVH and TO provided reagents for the experiments.

Writing the manuscript: MN with contributions from SB, LK, RAH, TO, AOGC and MJ wrote the manuscript.

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Figures and figure legends

Figure 1

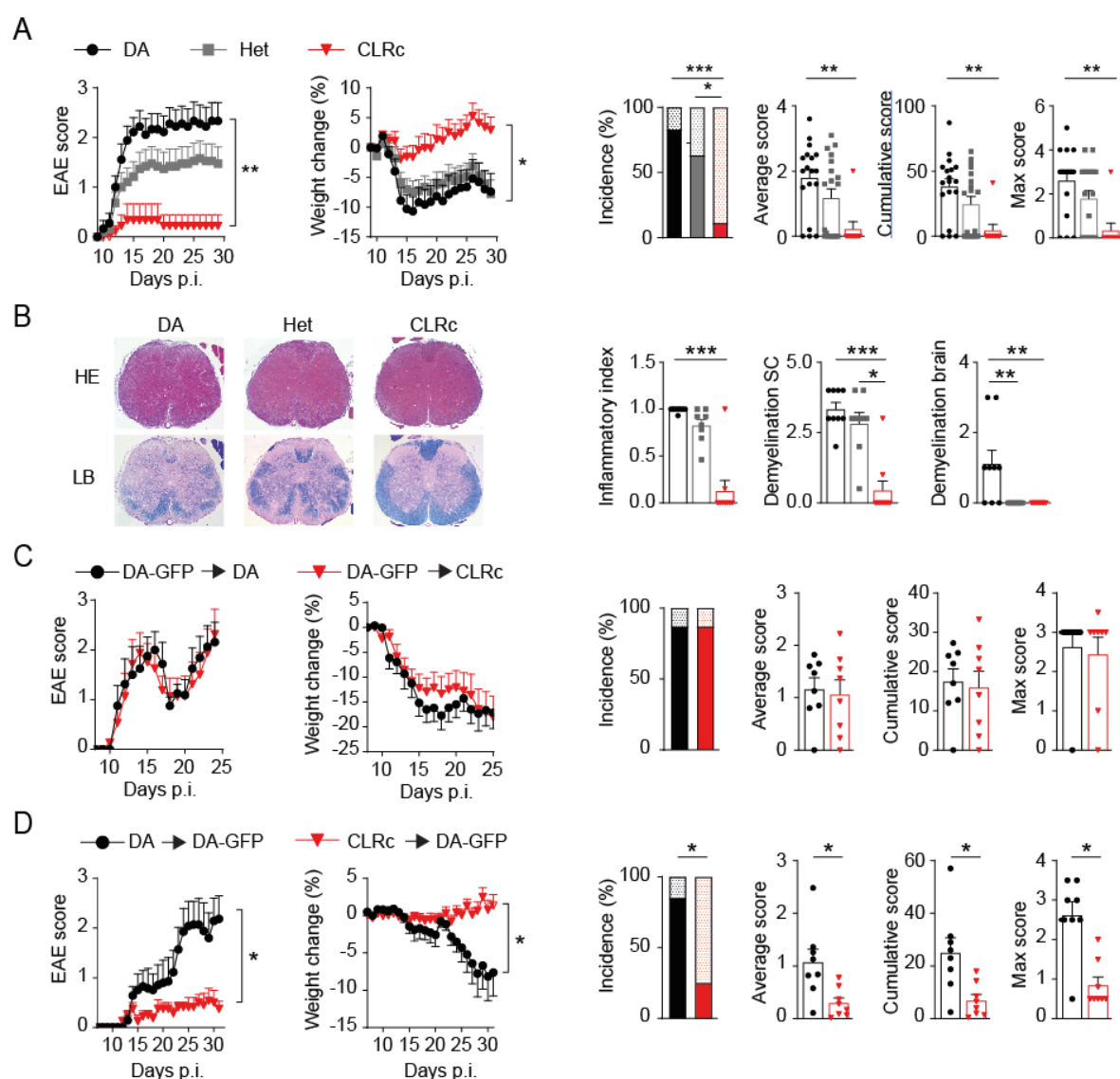


Fig 1. CLRC are protected from EAE in a peripheral immune cell-dependent manner.

Homozygous (CLRC) and heterozygous (Het) congenic rats and their littermate DA controls were immunized with MOG and followed for signs of disease. **(A)** Clinical signs of EAE and disease parameters in DA littermate controls ($n=18$), Het ($n=19$) and CLRC rats ($n=9$) (representative of three experiments). For EAE incidence, the upper dotted bars represent unaffected rats whereas the lower plain bars represent affected rats. **(B)** Histopathological analysis of spinal cord (sc) at day 29. *Left*, representative images of H&E and Luxol fast blue (LB) stainings (magnification 40X). *Right*, quantification of inflammation and demyelination for DA ($n=9$), Het ($n=8$) and CLRC rats ($n=9$). **(C)**,

D) Lethally irradiated rats were transplanted with bone marrow (BM) from donor animals, reconstituted for two months and then immunized with MOG. Clinical signs of EAE and disease parameters were assessed in **(C)** DA or CLRc recipient rats transplanted with DA-GFP BM (DA-GFP -> DA ($n=8$) or DA-GFP -> CLRc ($n=8$)) and **(D)** DA-GFP recipients transplanted with DA or CLRc BM (CLRc -> DA-GFP ($n=8$) and DA -> DA-GFP ($n=7$)). Data are presented as mean $\pm$ SEM. The following statistical tests were used: 1-way ANOVA with Dunnett's Multiple Comparison Test (**A**, for area under curve (AUC) of clinical EAE and weight change), Kruskal-Wallis test with Dunn's Multiple Comparison Test (**A**, for average, cumulative and max EAE score; **B**), Unpaired 2-tailed t-test (**C-D**, for AUC of clinical EAE and weight change), Mann-Whitney U test (**C-D**, for average, cumulative and max EAE score) and Chi-square test (**A**, **C-D**, for EAE incidence). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2

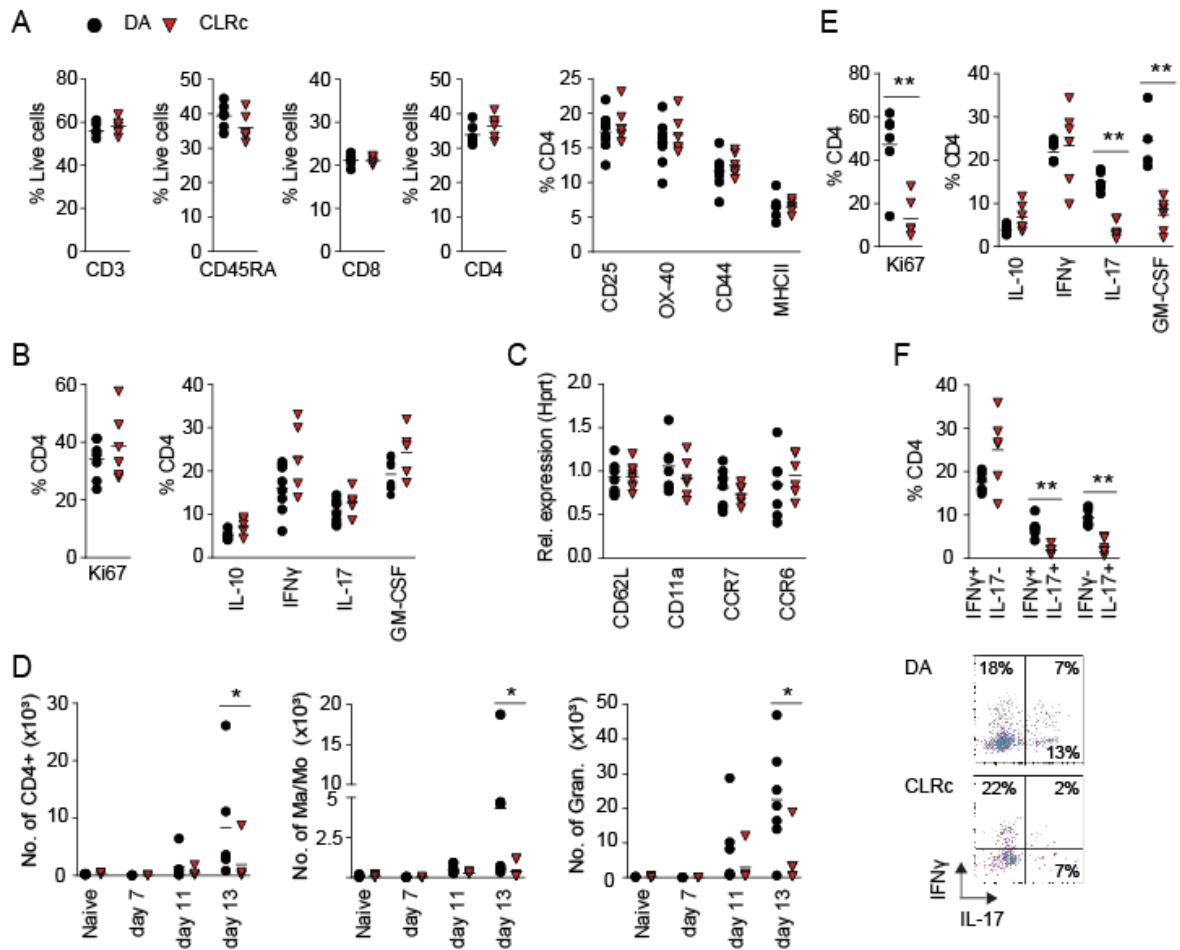


Fig. 2. Modulation of T cell activation in the CNS, but not in the periphery, underlies the protective CLRc phenotype. Homozygous CLRc rats and littermate DA controls were immunized with MOG and phenotyped during the disease course. Characterization of draining lymph node (dLN) and CNS infiltrating cells. **(A)** Frequency of total T cells (CD3), B cells (CD45RA), CD8⁺ and CD4⁺ T cells at day 7 p.i. in dLNs of DA ($n=6-8$) and CLRc ($n=6$) rats. Frequency of activation markers on CD4⁺ T cells (representative of four experiments). **(B)** Ki67 expression and cytokine production at day 7 p.i. in CD4⁺ T cells from dLN of DA ($n=7-8$) and CLRc ($n=5-6$) rats following 72 h in vitro MOG stimulation (representative of three experiments). **(C)** qPCR for *Cd62l*, *Cd11a*, *Ccr7* and *Ccr6* in CD4⁺ T cells sorted from dLNs of DA ($n=6$) and CLRc ($n=6$) rats day 7 p.i. in dLNs (representative of two experiments). **(D)** Number of infiltrating CD4⁺ T cells, macrophages/monocytes (Ma/Mo) and granulocytes in spinal cord of naïve DA and CLRc rats ($n=12$ and $n=12$) and at 7 ($n=3$ and $n=3$), 11 ($n=6$ and $n=5$) and 13 days ($n=7$ and $n=5$) post immunization (representative of two experiments). **(E)**

and (F) Characterization of proliferation and cytokine production in infiltrating cells isolated from spinal cord on day 13 p.i. stimulated in vitro with PMA/Ionomycin/BrefeldinA for 5 h in CLRC rats ($n=6$) and DA rats ($n=6$) (representative of two experiments). Data are presented as mean $\pm$ SEM. All comparisons were done with Mann–Whitney U test. $*P < 0.05$; $**P < 0.01$.

Figure 3

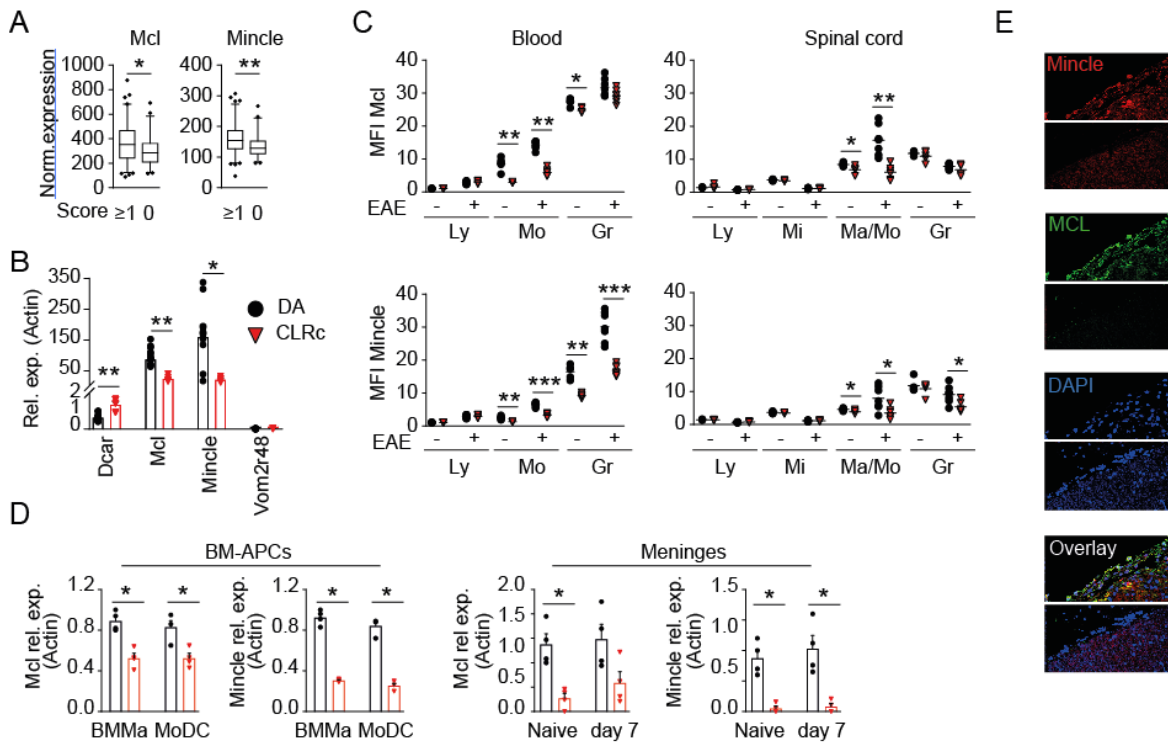


Fig. 3. CLRC regulates expression of *Mcl* and *Mincl* in monocytes and macrophages. (A) Expression of *Mcl* and *Mincl* assessed by microarray in spleens of (DAxPVG)xDA backcross rats subjected to EAE, affected ($n=95$, score ≥ 1) and non-affected ($n=51$, score 0). (B) Gene expression in DA ($n=6$) and CLRC ($n=6$) spleens determined by qPCR (representative of two experiments). (C) Flow cytometry analysis of cells isolated from blood and spinal cord of naive DA ($n=5$) and CLRC ($n=5$), as well as DA ($n=7$) and CLRC ($n=6$) rats at day 13 p.i. assessing *Mcl* and *Mincl* protein expression by mean fluorescence intensity (MFI) (representative of two experiments). Ly=Lymphocyte, Mi=Microglia, Mo/Ma=Monocyte/Macrophage, Gr=Granulocyte. (D) qPCR analysis of *Mcl* and *Mincl* expression in BMMa and MoDCs (BM-APCs) derived in vitro and meninges from naïve and 7 days p.i. rats of DA ($n=4$) and CLRC ($n=4$) (representative of two experiments). (E) Immunofluorescent staining of rat spinal cord 11 days p.i. Representative images of staining for *Mcl*, *Mincl* and nuclei (DAPI) (magnification 40X). Data are presented as mean $\pm$ SEM or box-plots with whiskers representing 5-95 percentile. All comparisons were done with Mann-Whitney U test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 4

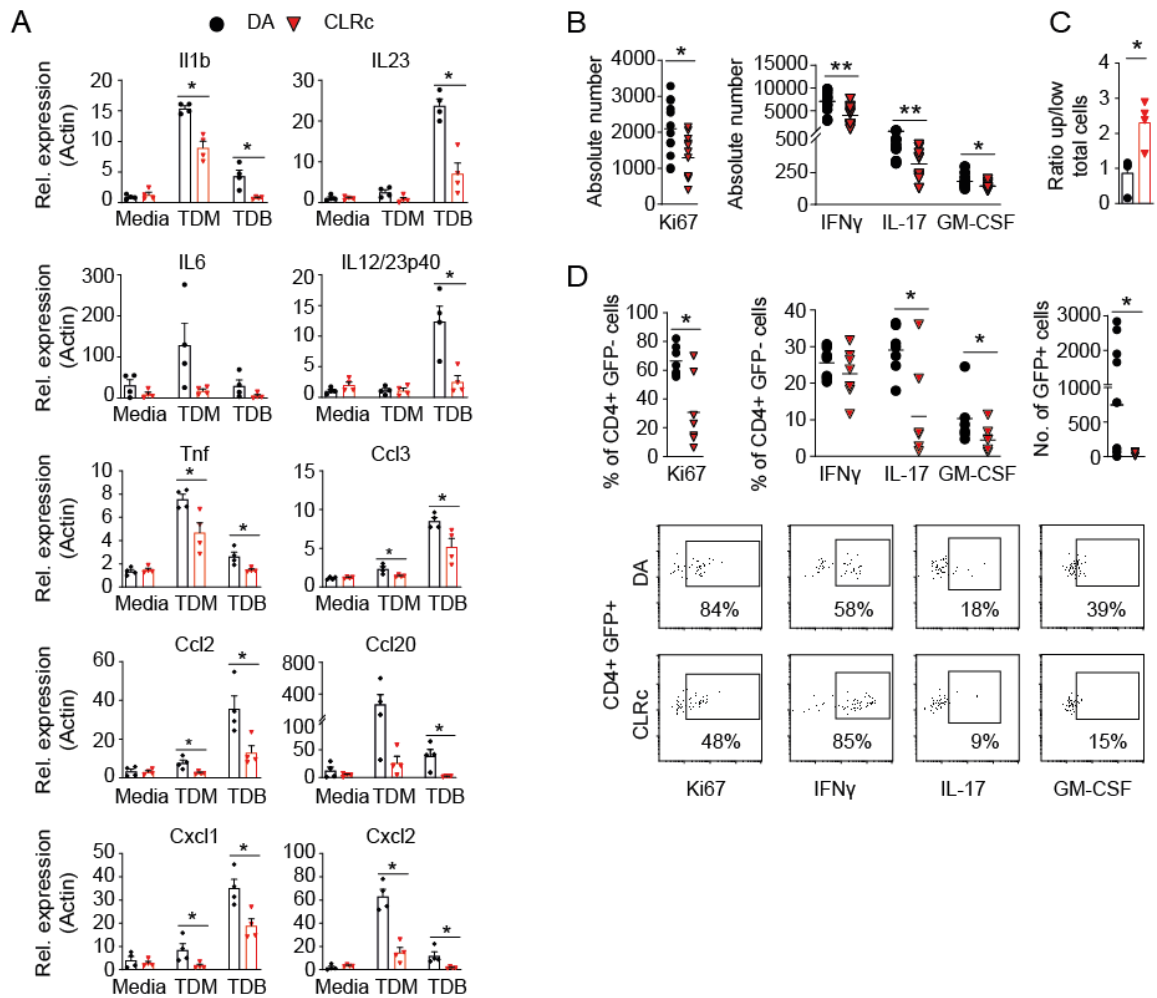


Fig. 4. Attenuated response to Mcl/Mincle stimulation in CLRC BMMA resulting in altered CD4⁺ T cell extravasation and activation. (A) Bone marrow derived macrophages (BMMA) from DA ($n=4$) and CLRC ($n=4$) rats were stimulated with receptor-specific ligands (TDM and TDB) for 18h (representative of three experiments). qPCR analysis of genes downstream of the Mcl/Mincle pathway. (B) MOG-specific CD4⁺ effector cells were reactivated for 4 days with DA ($n=6$) or CLRC ($n=6$) BMMA at a ratio of 40:1 (T cell:BMMA) in the presence of MOG peptide. Flow cytometry analysis of CD4⁺ T cell assessing cytokine production and proliferation (representative of two experiments). (C) Transendothelial extravasation of MOG-specific CD4⁺ effector cells towards DA ($n=4$) or CLRC ($n=4$) BMMA. Flow cytometry analysis of CD4⁺ T cell assessing transmigration (representative of two experiments). (D) Adoptive transfer of GFP⁺ MOG-specific CD4⁺ effector cells injected i.v. into DA ($n=7$) or CLRC ($n=7$) recipients 6 days p.i. (representative of two experiments).

Characterization of proliferation and cytokine production in both GFP⁻ as well as GFP⁺ infiltrating cells isolated from spinal cord on day 13 p.i. stimulated in vitro with PMA/Ionomycin/BrefeldinA for 5 h. Data are presented as mean $\pm$ SEM. All comparisons were done with Mann–Whitney U test. * P < 0.05; ** P < 0.01.

Figure 5

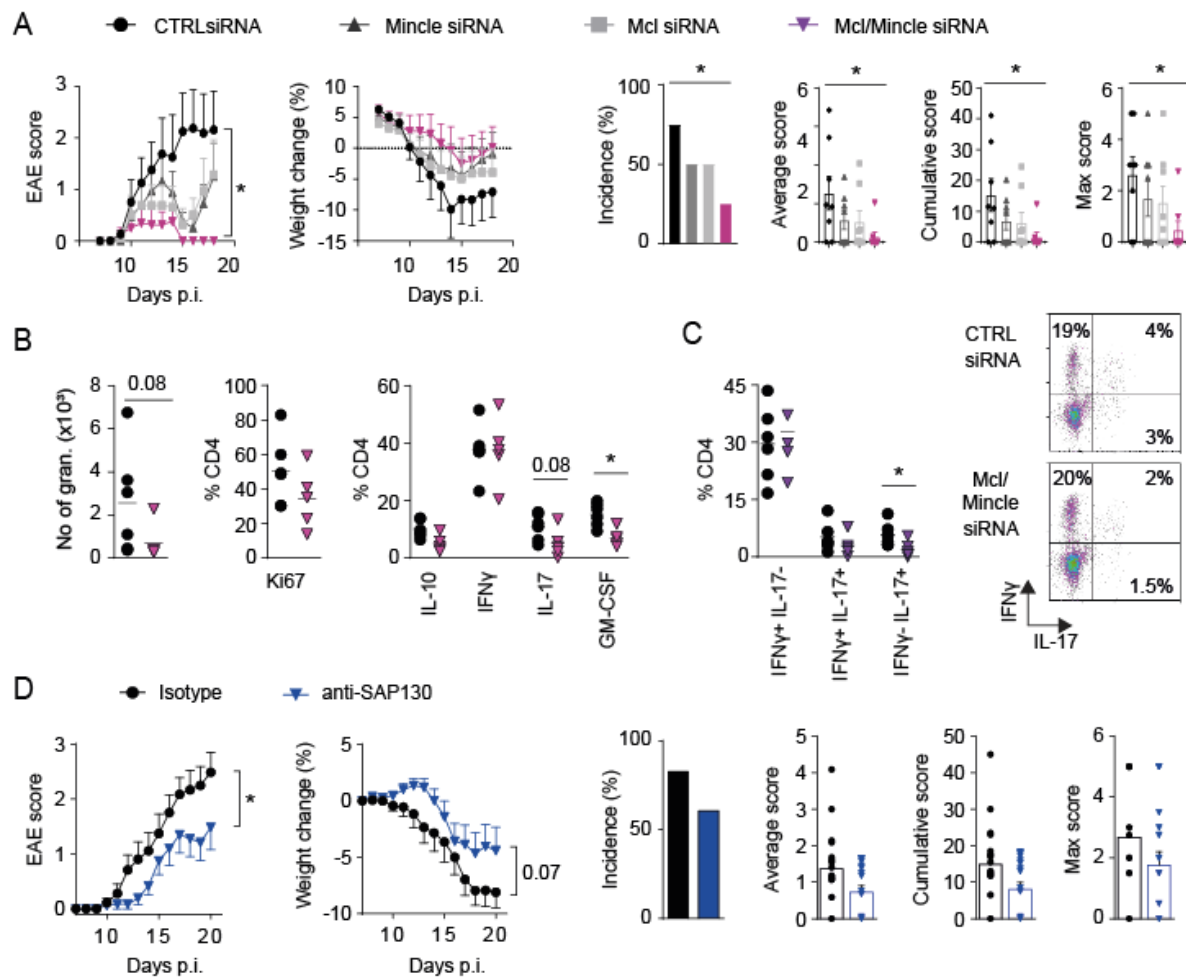


Fig. 5. CNS-specific Mcl/Minle silencing, as well as blockage of the endogenous ligand SAP130, protects from EAE. (A) MOG-immunized rats injected with *Mcl* ($n=8$), *Minle* ($n=8$), *Mcl/Minle* ($n=8$) or scrambled control siRNA ($n=8$) i.t. and i.c. at 7, 9 and 12 days p.i. were followed for clinical signs of EAE (representative of three experiments). (B) Characterization of proliferation and cytokine production of infiltrating cells isolated from spinal cord day 12 p.i., stimulated in vitro with PMA/Ionomycin/BrefeldinA for 5 h (proliferation and cytokine production) in rats injected with *Mcl/Minle* ($n=5$) or scrambled control siRNA ($n=6$) and (C) quantification of IFN γ - and IL-17-producing CD4 $^{+}$ T cells (representative of two experiments). (D) Clinical signs of EAE and disease parameters in DA rats treated with anti-SAP130 ($n=18$) or rabbit IgG isotype control ($n=18$) antibody i.t. and i.c. on days 2 and 7 p.i. (two pooled experiments). Data are presented as mean $\pm$ SEM. The following statistical tests were used: 1-way ANOVA with Dunnett's Multiple Comparison Test (A, for

area under curve (AUC) of clinical EAE and weight change), Mann–Whitney U test (**A-D**, for average, cumulative and max EAE score, **B** and **C**), Unpaired 2-tailed t-test (**D**, for AUC of clinical EAE and weight change) and Chi-square test (**A** and **D**, for EAE incidence). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 6

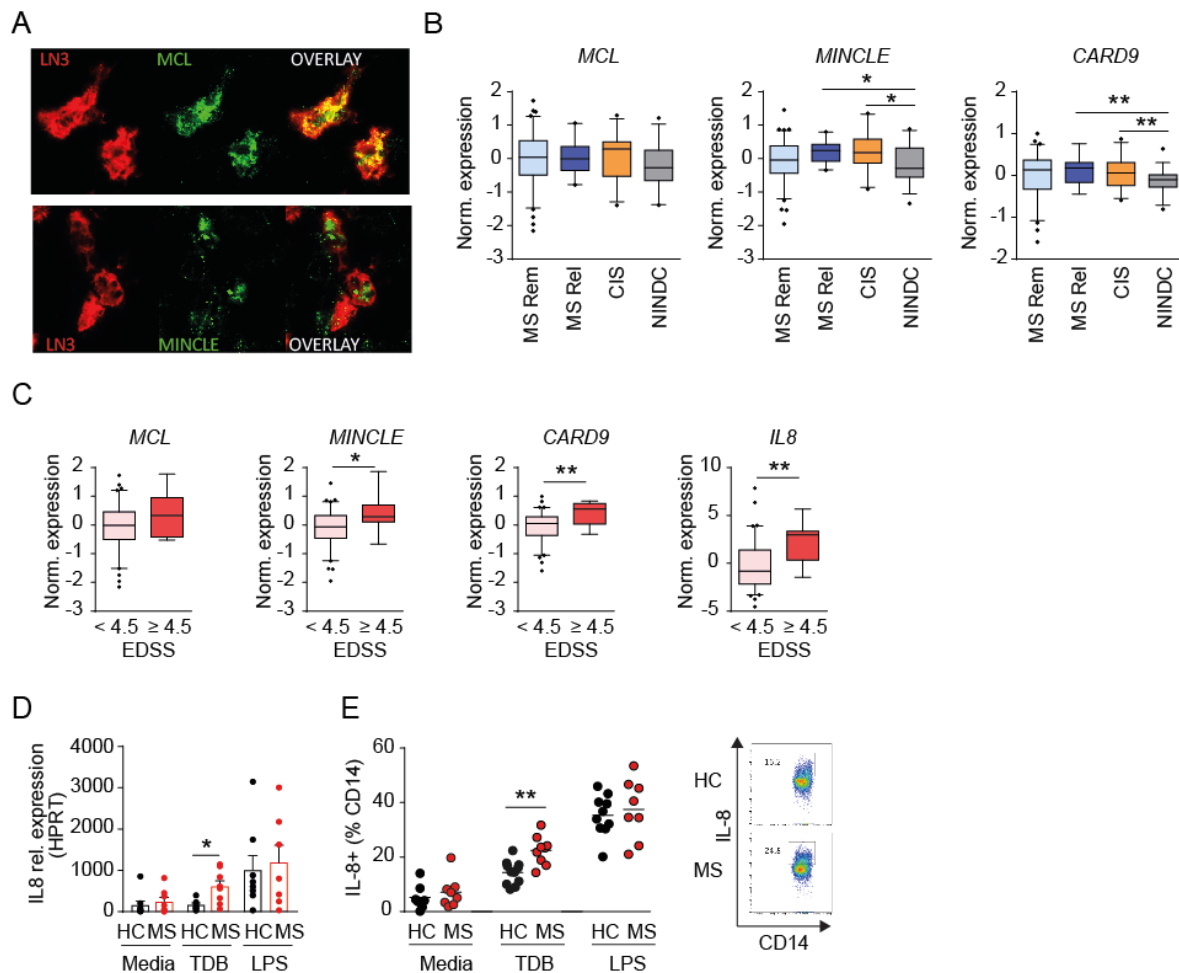


Fig 6. The MCL/ MINCLE signaling pathway is upregulated in MS patients and correlates with disease activity and progression. (A) Group representative immunofluorescent staining of MS lesions. MCL or MINCLE (green), HLA-DR expression (LN3, red), 40X magnification. (B-C) Gene expression analysis of PBMC from MS patients and non-inflammatory neurological disease controls (NINDCs) using RNA-sequencing. (B) Expression of *MCL*, *MINCLE* and *CARD9* comparing MS patients in remission ($n=73$), MS patients in relapse ($n=14$), CIS patients ($n=28$) and NINDCs ($n=36$). (C) Expression of *MCL*, *MINCLE*, *CARD9* and *IL8* according to Expanded Disability Status Scale (EDSS) score. (D) PBMC from RRMS patients in relapse ($n=9$) and healthy controls ($n=11$) were stimulated in vitro with either TDB, LPS or media. qPCR analysis of *IL8* expression of adherent CD14⁺ fraction after 24 h stimulation. (E) Flow cytometry analysis of IL-8 production gated on CD14⁺ monocytes after 48 h stimulation. Data are presented as mean $\pm$ SEM or box-plots with whiskers representing 5-95 percentile. The following statistical tests were used: 1-way ANOVA with Dunnett's

Multiple Comparison Test (**B**), Unpaired 2-tailed t-test (**C**) and Mann-Whitney U test (C and D). * $P < 0.05$; ** $P < 0.01$.